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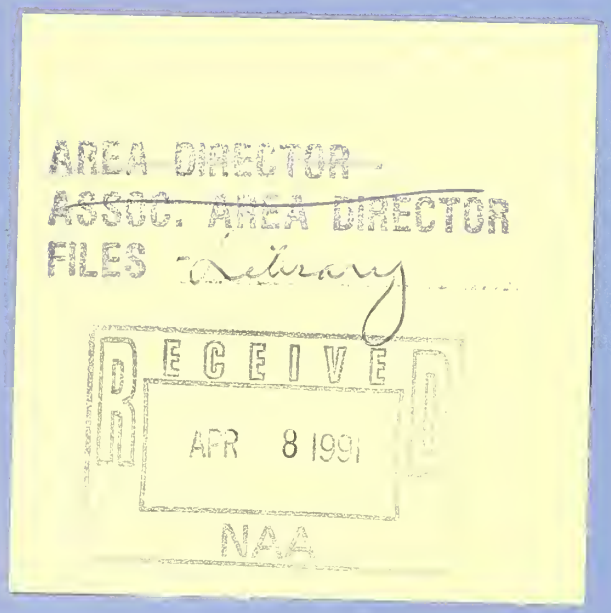

PREFACE

The United States Department of Agriculture Workshop on Virus-Thrips-Plant Interaction of Tomato Spotted Wilt Virus, was held April 18-19, 1990 at the Beltsville Agricultural Research Center, Beltsville, Maryland.

In recent years, widespread infections of tomato spotted wilt virus among field and horticultural crops have attracted much attention among entomologists, plant pathologists and extension workers from research communities and crop industries. Two conferences sponsored by the American Floral Endowment and numerous regional meetings were held to assess the problems and discuss current research. None of these meetings has been fully documented through publications.

Because of the rapid developments in tomato spotted wilt virus research, and the large number of unanswered questions that should be studied cooperatively, the Florist and Nursery Crops has conducted a two-day workshop specifically focused on pathogen-vector-host plant relationships of tomato spotted wilt virus. Topics discussed included biology of thrips vectors, biological association of the virus with thrips vectors, monitoring the virus in thrips vectors and in plants, as well as biology and molecular biology of the virus. The workshop brought together experts because of their knowledge of specific subjects and recent contributions in their specialized fields. The conference provided the opportunity to exchange up-to-date published and unpublished information and to develop new cooperation among researchers from around the world.

The Florist and Nursery Crops Laboratory, Plant Sciences Institute, hosted the Workshop and many people contributed to its success. The organizing committee: Dr. H. T. Hsu, Dr. R. H. Lawson, Dr. J. C. Locke, Peggy Hall, Judith Thompson and Dr. J. W. Neal, Jr., did an outstanding job on local arrangements and hospitality for all of the participants.

We gratefully acknowledge the support of Paul Ecke Poinsettias, Encinitas, California and Yoder Brothers, Inc., Alva, Florida that helped make this Workshop possible.

Hei-ti Hsu and Roger H. Lawson
Proceedings Editors
Tomato Spotted Wilt Virus Workshop

LIST OF PARTICIPANTS

Dr. Gunther Adam	Deutsche Sammlung von Mikroorganismen Arbeitsgruppe Pflanzeviren Messeweg 11/12 B-3300 Braunschwieg Federal Republic of Germany
Dr. Wayne R. Allen	Agriculture Canada, Research Station Vineland Station Ontario, Canada LOR 2E0
Dr. Joseph Begley	Yoder Brothers, Inc. P. O. Box ;68 Alva, Florida 33920
Ms. Jo-Ann Bentz	USDA, ARS, Florist & Nursery Crops Lab. Rm. 10, Bldg. 470, BARC-East Beltsville, Maryland 20705
Dr. James H. Blake	Clemson University Department of Plant Pathology & Physiology Clemson, South Carolina 29634-0377
Dr. A. Bruce Broadbent	Agriculture Canada, Research Station Vineland Station Ontario, Canada LOR 2E0
Dr. Larry G. Brown	Florida Department of Agriculture Plant Industry Division P. O. Box 1269 Gainesville, Florida 32602
Dr. John Cho	University of Hawaii Department of Plant Pathology Maui Branch P. O. Box 269 Kula, Hawaii 96790
Dr. Edwin Civerolo	USDA, ARS, National Program Staff Rm. 228, Bldg. 005, BARC-West Beltsville, Maryland 20705
Dr. Margery Daugherty	Long Island Horticultural Research Laboratory 39 Sound Avenue Riverhead, New York 11901
Dr. Robert F. Davis	Agdia, Inc. 30380 County Road 6 Elkhart, Indiana 46514

Dr. Ethel Dutky	University of Maryland Department of Botany College Park, Maryland 20742
Dr. Peter de Haan	Agricultural University Deparment of Virology P. O. Box 8046 6700 EM Wageningen, The Netherlands
Dr. Joseph E. Funderburk	North Florida Research and Education Center Route 3, Box 4370 Quincy, Florida 32351-9529
Dr. Abed Gera	Agricultural Research Organization Institute of Plant Protection P.O.B. 6, Bet Dagan 50250 Israel
Dr. Thomas L. German	University of Wisconsin Department of Plant Pathology 1630 Linden Drive Madison, Wisconsin 53706
Dr. Frederick Gildow	Pennsylvania State University Department of Plant Pathology University Park, Pennsylvania 16802
Dr. Rob W. Goldbach	Agricultural University Deparment of Virology P. O. Box 8046 6700 EM Wageningen, The Netherlands
Dr. Dennis Golsalves	Cornell University-New York State Agricultural Experiment Station Department of Plant Pathology Geneva, New York 14456
Dr. Mary Hausbech	Pennsylvania State University Department of Plant Pathology University Park, Pennsylvania 16802
Dr. Hei-ti Hsu	USDA, ARS, Florist & Nursery Crops Laboratory Rm. 208, Bldg. 004, BARC-West Beltsville, Maryland 20705
Dr. Ronald K. Jones	North Carolina State University Department of Plant Pathology Box 7616 Raleigh, North Carolina 27695-7616

Dr. Mike Klopmeyer	George J. Ball, Inc. P. O. Box 335 West Chicago, Illinois 60185
Dr. Roger H. Lawson	USDA, ARS, Florist & Nursery Crops Laboratory Rm. 208, Bldg. 004, BARC-West Beltsville, Maryland 20705
Dr. Anne Lemmetty	Agricultural Research Centre of Finland Institute of Plant Protection SF-031600 Jokioinen Finland
Dr. Richard K. Linquist	Ohio Agr. R & D Center Department of Entomology Wooster, Ohio 44691
Dr. James C. Locke	USDA, ARS, Florist & Nursery Crops Laboratory Rm. 208, Bldg. 004, BARC-West Beltsville, Maryland 20705
Dr. Edgar Maiss	Deutsche Sammlung von Mikroorganismen Arbeitsgruppe Pflanzeviren Messeweg 11/12 B-3300 Braunschweig Federal Republic of Germany
Dr. Ronald F. L. Mau	University of Hawaii Department of Entomology 3050 Maile Way Honolulu, Hawaii 96822
Dr. Larry L. McDaniels	American Type Culture Collection 12301 Parklawn Drive Rockville, Maryland 207852
Dr. Forrest L. Mitchell	Texas Agricultural Experiment Station P. O. Box 292 Stephenville, Texas 76401
Dr. James W. Moyer	North Carolina State University Department of Plant Pathology Box 7616 Raleigh, North Carolina 27695-7616
Dr. John W. Neal, Jr.	USDA, ARS, Florist & Nursery Crops Laboratory Rm. 208, Bldg. 004, BARC-West Beltsville, Maryland 20705
Dr. Ronald D. Oetting	Georgia Expt. Station Department of Entomology Experiment, Georgia 30212

Dr. Dick Peters	Agricultural University Deparment of Virology P. O. Box 8046 6700 EM Wageningen, The Netherlands
Dr. James F. Price	GCREC 5007 60th St. East Bradenton, Florida 34203
Mr. Jose Manuel Quiros	Linda Vista, S.A. Apartado 100-7050 Cartago Costa Rica
Dr. D. V. R. Reddy	International Crops Research Institute for Semi-Arid Tropics Pantanchero P. O. Andhra, Pradesh 502 324, India
Dr. Karen L. Robb	Cooperative Extension Farm and Home Advisors Office 5555 Overland Avenue, Building 4 San Diego, California 92123
Dr. John L. Sherwood	Oklahoma State University Department of Plant Pathology Stillwater, Oklahoma 74078
Dr. Donald Smith	Texas Agricultural Experiment Station P. O. Box 292 Stephenville, Texas 76401
Dr. James W. Smith, Jr.	Texas A & M University Biological Control Center Entomology Department College Station, Texas 77843
Dr. Judy St. John	Plant Sciences Institute USDA, ARS Rm. 425, National Agricultural Library Beltsville, Maryland 20705
Dr. Chester Sutula	Agdia, Inc. 30380 County Road 6 Elkhart, Indiana 46514

Dr. James F. Tammen	Institute for the Advancement of Floriculture 257 S. Pugh Street State College, Pennsylvania 16801
Dr. Jane C. Trolinger	Yoder Brothers, Inc. P. O. Box 68 Alva, Florida 33920
Dr. Diane E. Ullman	University of Hawaii Department of Entomology 3050 Maile Way Honolulu, Hawaii 96822
Dr. Bert van Blokland	Royal Sluis Postbox 22, 1600 AA Enkhuizen, Holland

CONTENTS

AN OVERVIEW OF TOMATO SPOTTEI WILT VIRUS

D. Peters, A. C. de Avila, E. W. Kitajima, R. de O. Resende, P. de Haan and R. W. Goldbach

ABSTRACT

The biology and the molecular properties of TSWV are discussed with respect to the diversity demonstrated by the various virus isolates.

INTRODUCTION

'Spotted wilt' was first observed in Australia in 1915 (Brittlebank, 1919) and was shown to have a viral etiology by Samuel et al. (1930). To date tomato spotted wilt virus (TSWV) is found in many other regions, especially those with a (sub)tropical climate, and may have a worldwide distribution (Best, 1968; Francki & Hatta, 1981). The virus is also the agent of diseases known as Kat river disease, makhorka tip chlorosis, pineapple side rot, pineapple yellow spot, tobacco and tomato carcova, tobacco kromnek, tomato bronze leaf and vira cabeca (Francki & Hatta, 1981). Although TSWV had a large interest to virologists, due to the serious diseases it can cause, it remained for years poorly characterized in terms of physical and molecular properties. This was mainly due to its instability in vitro which makes it often difficult to purify the virus for critical studies. The recently evoked interest, caused by the rapid spread of TSWV over the Northern hemisphere by Frankliniella occidentalis, by the molecular techniques available nowadays, and by the production of some highly sensitive antisera in a few laboratories, has considerably extended our knowledge on the virus in the last few years. This conference on TSWV presents many of these recent findings.

TRANSMISSION BY THRIPS

TSWV is transmitted by members of the Thripidae (Order: Thysanoptera). They include Frankliniella fusca (tobacco thrips), F. occidentalis (western flower thrips), F. schultzei (common blossom thrips), Scirtothrips dorsalis (chilli thrips) and Thrips tabaci (onion thrips). The virus is acquired only during larval stages. Larvae which acquire the virus early, can transmit it at the end of the last larval stage. Infectious adults transmit through their life. The transmission process cannot be expressed yet in terms of AAP_{50} or IAP_{50} (i.e. the period in which 50% of the thrips respectively acquire and transmit the virus). Also, the efficiency by which the various species transmit, and the effect of virus source and the plants infected on this efficiency have not been studied in-depth. Therefore, the epidemiology of the virus can hardly be described beyond terms of population size of the vector in the field and glasshouse. The virus has virtually been absent in Western Europe after the second World War, although its vector Thrips tabaci was present in small numbers. Failure to spread the virus by this vector has been correlated with the absence of males in Polish T. tabaci populations. Thrips of the T. tabaci 'type tabaci' populations, in which males also occur, seems to transmit TSWV, whereas those of the 'communis type' populations consisting of only females do not transmit the virus (Zaqirski, 1976). In the present Dutch population of this thrips species, males appear to be absent (Vierbergen, personal communication).

HOST RANGE

TSWV is well known for its broad host range. The last published report lists almost 250 susceptible plant species (Cho et al., 1987), whereas Peters (unpublished) assembled a list of nearly 400 susceptible species occurring in more than 50 families. More than one hundred species are recorded within the Solanaceae and Compositae.

Department of Virology, Agricultural University, Binnenhaven 11, P. O. Box 8045, 6700 EM Wageningen, The Netherland.

The number of plant species which are naturally infected is indeed impressive. It should be noted that this number is not only determined by host susceptibility but also by the large range of plant species which are accepted as hosts by the different thrips species transmitting the virus. The reported number of plant species which are naturally infected will certainly increase with intensification of agriculture in its broadest context and with further examination of wild plant populations. Studies on the artificial host range will also elucidate more susceptible plants. These studies will be more meaningful when the search is directed to species or cultivars which are not susceptible. Such species may become sources of useful resistance genes.

SYMPTOMATOLOGY

TSWV exhibits a wide range of virulence patterns in different plant species. In addition, great variation in symptom expression on a single host species is often observed and is a well accepted phenomenon. However, the first reports (Samuel et al., 1930) ascribed these frequent variations to environmental causes, especially to temperature and the physiological constitution of the host. The possibility that different strains would exist was only established after studying the agent causing tomato tip blight (Milbrath, 1939). Norris (1943) showed that this virus, originally thought to be a new one, was a strain of TSWV. Stable symptom expression by TSWV isolates can be obtained by serial passage through certain hosts (Norris, 1946). This author revealed the existence of five distinct strains which were placed in three groups. One strain, tip blight (TB), alone comprises one group characterized by its lethal action on almost all hosts. The N and R strains characterized by Norris produce several stunting and some necrosis. These strains were placed in second group and the M and VM strains in a third group. The strains forming the latter group are characterized by mild symptoms with little growth retardation.

Best and Gallus (1953) characterized six strains by serial transfer through three indicator plants. They did not notice any change in symptom expression over a period of years and accepted them as naturally occurring, genetically stable strains. The symptoms produced by these strains were classified as follows: (a) Three main groups according to the systemic symptoms produced on Lycopersicum esculentum; (b) Three groups by the type of local lesions produced on Nicotiana glutinosa; and (c) Two groups with respect to systemic invasion on N. tabacum.

Table 1. Differentiation of six TSWV strains by symptom expression on three differential hosts.

	Strains					
	A	B	C_1	C_2	D	E
Lycospersicon esculentum	a	a	b	b	a	c
Nicotiana glutinosa	d	d	e	f	d	f
N. tabacum cv Blue Pryer	g	h	h	h	h	h

a: severe necrosis along with formation of brown/purple pigment.
b: mild surface necrosis in the form of ringspots or parallel line pattern without pigmentation.
c: neither visible necrosis not pigmentation.
d: necrotic pigmented disc-shaped lesions.
e: yellow disc lesions with necrosis at the outer edge.
f: non-necrotic yellow discs.
g: no systemic invasion.
h: systemic invasion.

All strains reacted differently on these three hosts (Table 1). This description of symptoms can be considered a first attempt to classify TSWV isolates. Although symptoms are often described meticuously by most authors in recent literature, references are unfortunately not made to the categories developed by Norris (1946), or Best and Gallus (1953).

CYTOPATHOLOGY

The virus invades all vegetative organs and tissues following systemic infection, including cells undergoing mitosis (Francki and Grivell, 1970). Ie (1973) detected virus in the endothelial tissue, but particles were not found in either tapetal cells or in pollen.

The particles observed in thin sections of infected cells are usually similar in structure and size as those found in dip preparations made from infected plant sap or purified preparations (Kitajima, 1965; Milne, 1970; Francki and Grivell, 1970; Ie, 1971; Paliwal, 1976). However, a recent Dutch isolate, denoted H7, produces in addition to spherical particles also oval and bacilliform particles as shown in thin sections. TSWV particles accumulate usually in clusters within the cistaernae of the endoplasmic reticulum (Fig. 1). The particles of some isolates can be found only scattered between these membranes. Whether this behavior reflects the number of particles formed or that this is a characteristic property of such a peculiar isolate is still an open question.

In addition to mature virus particles, the cytoplasm of infected cells may contain other distinct virus-induced structures. One type is characterized by dense masses often embedded in a stroma of a less compact composition (Fig. 1). These structures are intimately surrounded by ribosomes and occur in the vicinity of cistaernae with the virus particles. The dense masses have rugged outlines and are usually somewhat larger in size than the inner diameter of the virus particles (Ie, 1971). A periodicity of 5 nm can be observed in these dense masses. The nature of the dense masses was not clear for sometime. They have been considered to be viroplasms in the past (Milne, 1970; Ie, 1971). However, the isolation of defective isolates may require reinterpretation of their nature. Ie (1982) observed these masses in cells devoid of any characteristic virus particle and concluded that the isolates studied were defective, i.e. incapable of forming mature virus particles.

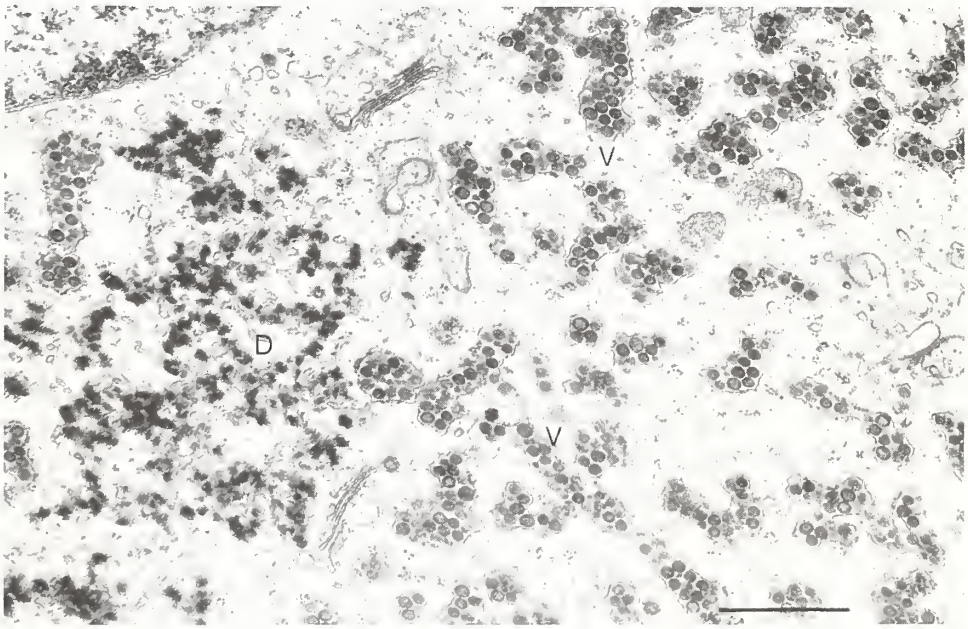

Fig. 1. Mesophyll cell of Nicotiana tabacum cv Samsun NN infected with tomato spotted wilt virus (TSWV). The cytoplasm contains clusters of enveloped particles (V) within the dilated cistaernae of the endoplasmic reticulum and a complex of dense masses (D). (Bar present 1 um).

Isolation of infectious material suggested that the dense masses have to be considered as aggregates of nucleocapsids of defective viruses (Verkleij and Peters, 1983). Association of nucleocapsid protein with these masses could be confirmed by immunogold labelling using anti-N immunoglobulins (Fig. 2). Many questions with respect to the shift of virus particles to defectiveness have not been answered but it is clear that it happens by alteration of lack of selective pressures, e.g. thrips transmission. Kitajima et al. (in preparation) noticed that in Nicotiana rustica plants the amount of dense masses in the infected cells seems to increase with the number of mechanical transfers made. Indeed, it is unlikely that thrips will transmit the defective forms, since the virus may have then lost its envelop during evolution.

In addition to the structures described above, elongated filamentous materials about 10 nm in diameter has been found in the cytoplasm of infected cells (Kitajima, 1965; Francki & Grivell, 1970; Law & Moyer, 1990). Two characteristic types can be distinguished. One type seems to consist of thin, but rather rigid rod-like material, often in paracrystalline arrays (Fig. 3). Another form, tubular and more flexuous, forming loose or bended parallel arrays, appears as large aggregates in the cytoplasm (Fig. 4). These filamentous structures occur in about half of the isolates studied so far by electron microscopy, and only one of the described types is then found in one host (Kitajima et al., in preparation).

Finally, it should be noted that most, if not all, isolates could not be distinguished cytomorphologically from each other by the appearance of dense masses and the way the particles accumulate in the cisternae.

VIRUS STRUCTURE

Electron micrographs of purified TSWV preparations contain roughly spherical particles (Black et al., 1963; van Kammen et al., 1966; Mohammen et al., 1973). In most preparations the particles appear to vary in diameter between 70 and 120 nm. They appear to have a unit membrane envelope with knob-like glycoprotein structures on their outer surface. The virus contains at least four different proteins, as internal nucleocapsid protein (N) of M_t 28000, two glycoproteins (G1 and G2) of 78 and 58K, respectively, and a large protein (in minor amounts) of an estimated M_t of 200 K (Mohamed et al., 1973; Tas et al., 1977a). In some preparations a fifth protein of 52K, also a glycoprotein (referred to as G2b), can be resolved (Tas et al., 1977a). It has not been established whether this is a distinct protein or a discrete degradation product of the G2 protein.

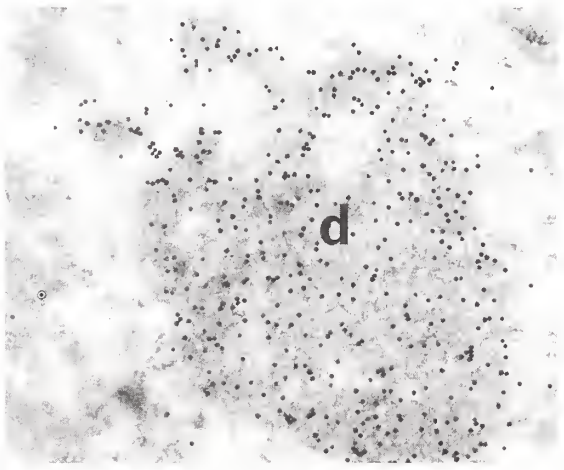

Fig. 2. Mesophyll cell of Datura stramonium infected with tomato spotted wilt virus showing a virus induced inclusion of dense masses (d) gold-labelled with anti-nucleocapsid protein antibodies.

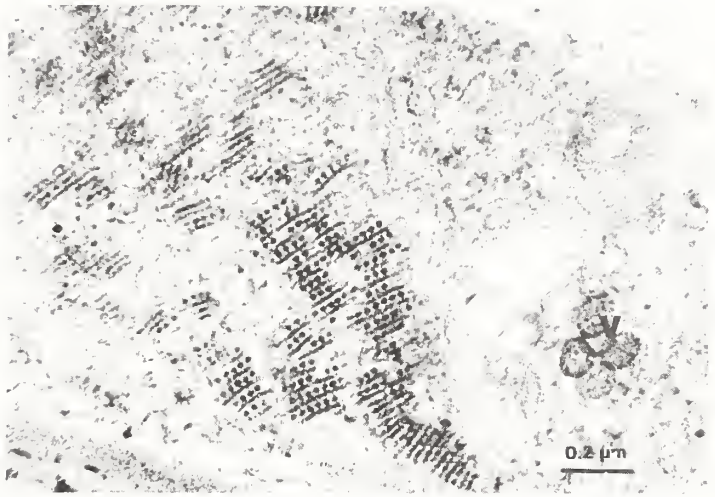

Fig. 3. Mesophyll cell of a tomato plant infected with tomato spotted wilt virus showing a few virus particles and an inclusion with elongated rigid filamentous material.

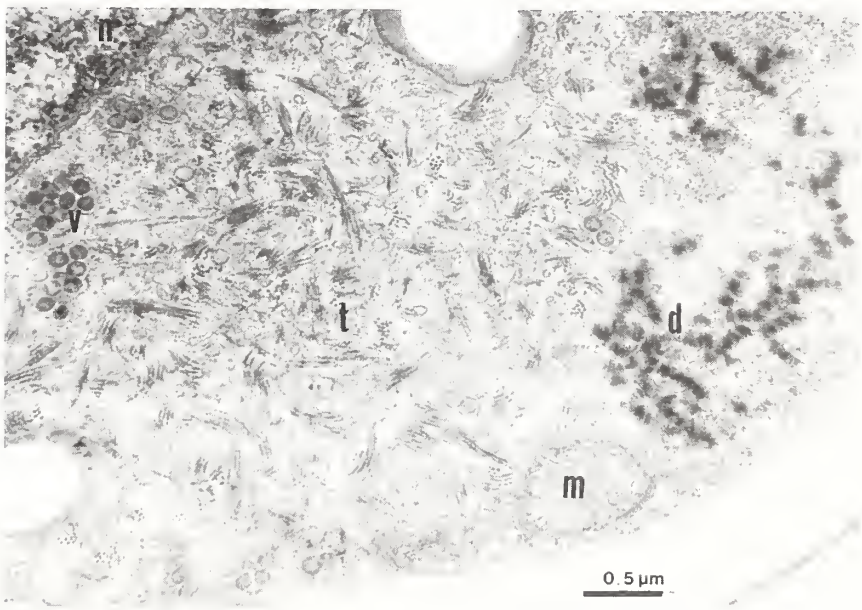

Fig. 4. Thin section of a Nicotiana rustica leaf mesophyll cell infected with tomato spotted wilt virus. A large inclusion of elongated tubular structures (f) appears in the cytoplasm between some clusters of virus particles (v) and groups of dense masses (d). (m=mitochondrion, n=nucleus).

All proteins seem to contain polysaccharide constituents (Mohamed et al., 1973; Tas et al., 1977a). Glycosylation of the G proteins could be expected, but it is more difficult to explain the association of carbohydrates with the N protein. Sometimes, a number of proteins, of which one is also glycosylated, are found in minor amounts in polyacrylamide gels, above the G1 protein band. They may be either contaminants of host origin, aggregates of one or more viral proteins or an unidentified structural protein of TSWV. It is obvious that more work is needed to determine the details of TSWV proteins composition; also the function of the proteins and their topographical position still await to be determined. Three different RNA

molecules, denoted S, M and L RNA have been found in purified preparations, which are not infectious. All segments appeared to be single stranded. For the Brazilian isolate CNPH1, the S RNA is 2916 nucleotides long (de Haan et al. 1990a). The M and L RNA of this isolate have not yet been completely sequenced, but their sizes have been estimated to be 5200 and 8200 nucleotides long (de Haan et al., 1989 and 1990b). The S RNA has an ambisense polarity and codes for two proteins, an N protein and a non-structural protein, denoted NSs (de Haan et al., 1990a). The M RNA is expected to specify the G1 and G2 proteins, whereas the L RNA, the viral transcriptase, may correspond to the observed L protein (de Haan et al., 1990b).

THE NUCLEOCAPSID COMPOSITION OF VARIOUS TSWV ISOLATES

TSWV contains three RNA segments which are encapsidated in nucleocapsids that form circular structures (Fig. 5). The length of these circles will be determined by the size of the RNA segments. After purification and analysis of the nucleocapsid fraction in a sucrose gradient three fraction are usually revealed (Fig. 6, see isolate A_1). These fractions, although pure fractions have not been obtained yet, are referred to as S, M and L depending on the descending position in a sucrose gradient after centrifugation and their RNA content.

Fig. 5. Micrograph of the nucleocapsids of TSWV spread by the Kleinschmidt technique.

Several features were observed by analyzing the ratio in which the nucleocapsids in different TSWV isolates occur. The sedimentation profiles of the nucleocapsid extracts of most TSWV isolates studied differed considerably from each other (Fig. 6). Each isolate seems to have its own characteristic profiles when the nucleocapsids are extracted from one and the same host. A second feature was observed when the isolate CNPH1 was propagated in N. rustica and Nicotiana benthamiana. The S, M and L nucleocapsid fractions were clearly discernable after analysis of the nucleocapsid extract from N. rustica. On the contrary, the S fraction was almost absent in an extract prepared from infected N. benthamiana plants (Fig. 7). A fraction sedimenting slightly more rapidly than the M fraction was found. RNA analysis revealed equal amounts S RNA in the N. benthamiana and N. rustica extracts. This observation suggests that the S nucleocapsid might have formed a dimer, the theoretical position of which corresponds with the position in the gradient.

Finally, a different nucleocapsid profile was found when an isolate was serially transferred in two lines through different host plants. Four sedimentating fractions were found in one sub-isolate and three in the other (results not shown). The position of the M and L fractions were similar. The position of the S fraction differed in the gradient, whereas a new fraction between M and L appeared.

As cited above, the diameter of the virus particles differs in size. This can be explained by the incorporation of concatamerized nucleocapsids. The possibility that less or more nucleocapsids are incorporated in one particle than the three nucleocapsids containing the whole virus genome can also not be excluded. The failure to identify three size classes of nucleocapsids by estimating the length of the nucleocapsid circles using the Kleinschmidt method may also indicate that they may form concatamers.

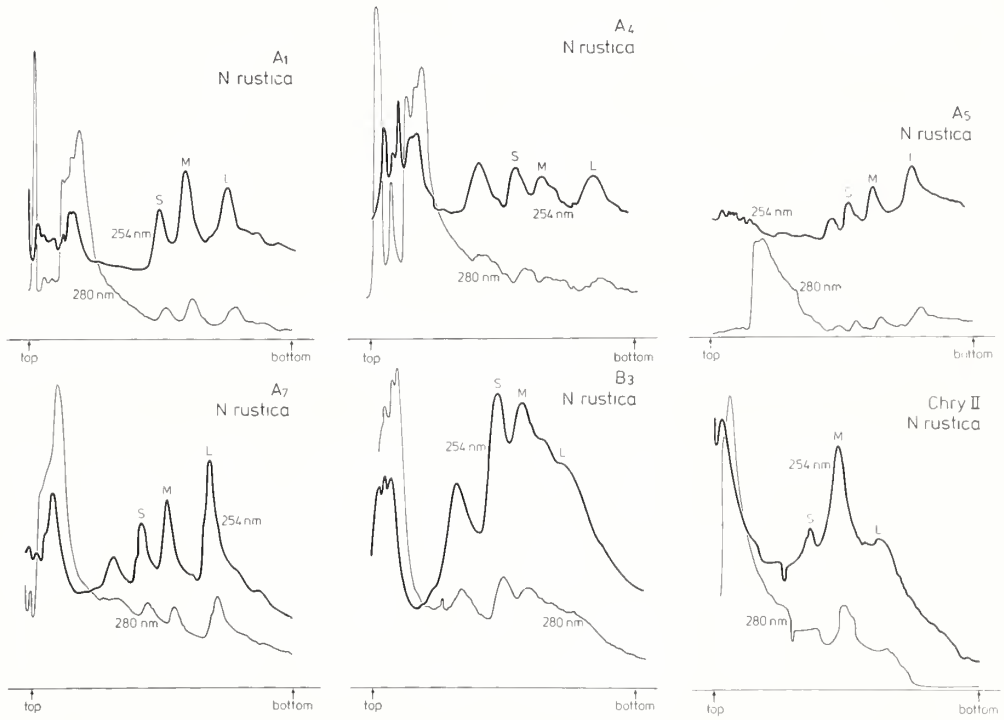

Fig. 6. Sedimentation profiles in a sucrose gradient of the nucleocapsid fraction of six different TSWV isolates propagated in Nicotiana rustica.

Rice stripe virus (RSV), maize stripe virus (MStV), rice grassy stunt virus (RGSV) and rice hoja blanca form the tenuivirus group. They also have been shown to contain circular nucleocapsids like TSWV (Ishiwaki et al., 1989). These viruses are associated with five (MStV) or four (RSV and RGSV) single stranded and double stranded RNA segments. The morphology of the nucleocapsids of these viruses suggests that their taxonomic position is rather close to that of TSWV.

GENETICS

In view of the observation that TSWV has a tripartite RNA genome, the possibility exists that new viruses may be generated in dual virus infections by reassortment of RNA segments. For Bunyaviridae intratypic and intertypic genetic reassortment has been demonstrated by using ts mutants of California serogroup viruses (Bishop and Beaty, 1988). Viruses representing different bunyavirus serogroups seem not to be able to generate recombinant viruses.

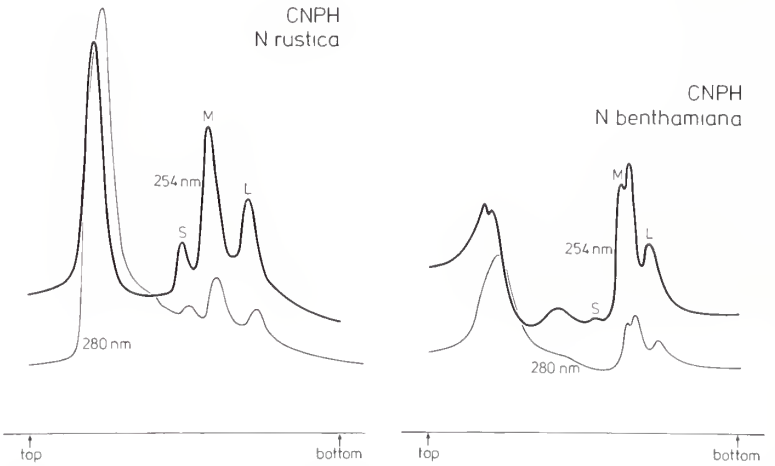

Fig. 7. Sedimentation profiles in a sucrose gradient of a nucleocapsid extract of the CNPH isolate after propagation in Nicotiana rustica or Nicotiana benthamiana.

Studies in which biological or physical properties could be assigned to genes on a particular segment of TSWV have not yet been made. However, Best and Gallus (1955) made some observations that were interpreted as a recombination of genetic information between two distinct isolates. After co-infection of two strains, denoted A and E, on tomato and N. glutinosa, a phenotypically distinct isolate was found. The authors concluded that the new isolate was a "recombinant" of the two original ones. Best (1961) has elaborated this study further. After having purified the parental strains by a single-diluton-at-limit-dilution technique, three new stable isolates were obtained. Two of the new strains, T_2 and R_3, had new characters not possessed by either parental strain A or E. The data can only be interpreted that the new strains arose as a result of an exchange of genetic determinants between the original strains A and E. In "back-crossing" with strains R_1 and E, strain A could be recovered while strain E appeared when he strains R_1 and A were used. The ideas and results of Best were not widely accepted, but with the present knowledge of TSWV reassortment of segments is the most plausible explanation. It should be noted that only one property, expression of symptoms, was used in his study. With our present knowledge, strains may be found with the required distinguishable properties to perform reassortment experiments to elucidate the genetics of this virus in more detail. Direct evidence for naturally occurring reassortment of viruses has not yet been reported. If it occurs, reassortment certainly can be a major contribution to the observed diversity of TSWV.

DETECTION AND DIAGNOSIS

TSWV has several properties by which it can readily be distinguished from other viruses. We subscribe to the view that the use of specific antiserum offers the most reliable criterion to identify plant viruses. Serology has not often been used to identify TSWV in the past. A strong evidence for its identity could be obtained by sap transmission tests to several selected host plants. Of these, Petunia seems to be one of the most useful because of its reaction with small brown or black local lesions on the inoculated leaves within two days. This species is less sensitive when the plants are grown under conditions of intense sunlight.

The virus has, among plant viruses, a unique morphology. The roughly spherical enveloped particles should readily allow identification by electron microscope in leaf dip preparations. However, the results are not always convincing because the particles tend to flatten and become distorted, especially when they are not fixed before staining. Fixation may prevent much of the distortion but even so it is often difficult to decide with confidence if a particle is that of TSWV. The particles often cannot readily be distinguished from some similar sized membranous host cell components. Immunogold labelling with virus specific antibodies, on the other hand, helps to confirm the identity of the virus particles in the extract.

Identification of TSWV in thin sections from tissues of infected plants is a more reliable method. With present knowledge the particles cannot be mistaken for those of any other plant virus, the presence of the dense masses (see above) in the infected cell constitutes an additional reliable characteristic of a TSWV infection.

Transmission by thrips, although a unique property, has not routinely been applied to identify the virus in laboratories. To prevent uncontrolled spread of thrips and virus through glasshouses, more elaborate rearing techniques, than is common practice for other plant virus vectors, are required.

The conspicuous lack of serology until very recently in the diagnosis of TSWV has been owed to the difficulties in obtaining sufficient amounts of pure antigen (Francki & Hatta, 1981). Thus far, three different techniques have been used in the serology of TSWV. Using gel diffusion test titers between 1/8 and 1/128 were found (Feldman & Boninsegna, 1968; Tsakirides & Gooding, 1972; Joubert et al., 1974; and Tas et al., 1977b), and in tube precipitin and ring test titers between 1/256 and 1/512 (Best & Hariharasubramanian, 1967; Paliwal, 1976). These techniques were not very sensitive with TSWV. They have often been applied with either purified virus preparations or undiluted extracts from infected plants.

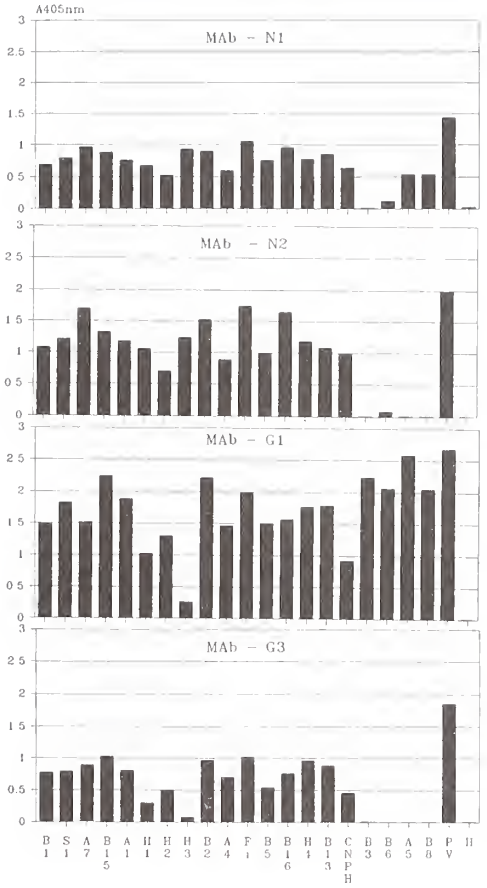

Fig. 8. Reactivity of two anti-nucleocpasid protein monoclonals (N1 and N2), and two anti-membrane protein monoclonals (G1 and G3) with a panel of 20 tomato spotted wilt virus isolates from different host and climate zones.

The development of ELISA has dramatically increased the success of serology in the detection of TSWV, both in (highly diluted) extracts from infected plants (Gonsalves & Trujkillo, 1986; Huguenot et al., 1990; and Resende et al., in preparation) and in thrips (Cho et al., 1988). Hence, success of serology of TSWV not only seems to depend on the quality of the antisera, but also on the serological techniques applied. Since ELISA is more sensitive than any other serological technique and can be more readily applied than the other methods discussed above for TSWV identification, it can be safely concluded that detection and diagnosis of this virus has basically been solved. The use of riboprobes (Huguenot et al., 1990) and cDNA probes (Ronco et al., 1989) has been advocated but are not yet widely applied in the identification and diagnosis of TSWV.

SEROLOGICAL VARIATION AMONG TSWV ISOLATES

ELISA has been introduced in the detection of TSWV by Gonsalves & Trujillo (1986). When tested in different ELISA formats TSWV appears to exhibit a great variation using antisera raised to different virus antigens (Wang & Gonsalves, personal communication). This has been confirmed in recent studies by Avila et al. (in preparation) in which the serological reactions of 20 TSWV isolates were analyzed in ELISA using polyclonal antibodies against isolate CNPH1 or against the nucleocapsid protein. Six monoclonals, two against the nucleocapsid protein and four against one of the G proteins,

presumably G1, were also incorporated in this study (Huguenot et al., 1990). The panel of TSWV isolates was divided by the polyclonal antisera into two groups. One group, reacting efficiently with the polyclonal antibodies, consisted of sixteen isolates, while the other group of four isolates reacted poorly with these polyclonal antisera. The sixteen isolates from the first group (Group I) also reacted with all MAbs, whereas the other four, forming serogroup II, were further differentiated by the MAbs (Fig. 8). The concepts of serogroup and serotype as defined for the serological classification of the Bunyaviridae (Bishop & Shope, 1979; Bishop & Beaty, 1988) have been introduced to classify TSWV serologically. A serogroup refers to antigenic relationships between the nucleocapsid protein as established with polyclonal antisera, while a serotype is based on serological differences between these proteins as found using a panel of monoclonal antibodies. A tentative proposal to classify TSWV in serogroups and types is summarized in Table 2. The Dutch isolate H7, already mentioned above, did not react with any of the available antisera or monoclonals in ELISA. From these results it is evident that TSWV can be divided into at least three serogroups and four serotypes (Table 2). Law and Moyer (1990) described a serological distinct isolate from Impatiens of TSWV, denoted TSWV-I. The nucleocapsid protein of this isolate was found serologically to be unrelated to that of the reference isolate in Western blot analysis using polyclonal antibodies. However, the G1 and G2 proteins were found to be related. Due to the different immunotechniques used it is not yet possible to classify isolate TSWV-I in one of the groups or types summarized in Table 2.

Table 2. Serogrouping of 21 TSWV isolates from various hosts, geographically different areas and climate zones by antiserum to purified preparations of the CNPH isolate, the nucleocapsid fraction of this virus and six monoclonals prepared to the N and G proteins of this virus.

Serum/MAb	Serogroup I Type I A1 A4 A7 B1 B2 B5 B13 B15 B16 F1 H1 H2 H3 H4 S1 CNPH	Serogroup II Type II B3 B6	Serogroup II Type III A5 B8	Serogroup? Type ? H7
Cv-PAb	+++	-+-	-+-	---
NuPAb	+++	-+-	-+-	---
MAb-N1	+++	---	+++	---
MAb-N2	+++	---	---	---
MAb-G1	+++	+++	+++	---
MAb-G2	+++	+++	+++	---
MAb-G3	+++	---	---	---
MAb-G4	+++	+++	+++	---

CONCLUSION

On one hand, TSWV had a unique position in the taxonomy of plant viruses and has therefore been described as the sole member of a monotypic group (Ie, 1970; and Matthews, 1982). On the other hand, it shares so many properties with the bunyaviruses that the virus may form a new genus in the family of the Bunyaviridae (de Haan et al. 1989; Milne & Francki, 1984). The name phythrivirus (Plant(=Phytos)-infecting, thrips-transmitted bunyavirus) has been proposed to the ICTV. In view of a similar circular nucleocapsid structure and the ambisense gene arrangement in S RNA of rice stripe virus (Kakutani et al., 1990) tenuiviruses may have a taxonomic position very close to TSWV.

With the present knowledge on TSWV, it has been clear that this pathogen exhibits a great diversity with respect to serological properties, symptom expression on different hosts, the formation of cytoplasmic inclusions, the rate in which the RNA segments are synthesized, and in the sedimentation profile of the nucleocapsid fraction. This variation may reflect the potential of this virus to respond rapidly to changing conditions and

different hosts. An example of this variability may be mentioned in the generation of isolates clearly different from the original isolate after serial passage and the generation of defective isolates. The potential of TSWV to change rapidly and to adapt to new hosts may explain the success of this virus to expand into the niche which has been occupied by F. occidentalis populations after its spread over the Rocky Mountains into the eastern USA and even more eastward into Europe.

REFERENCES

Best, R. J. 1961. Recombination experiments with strains A and E of tomato spotted wilt virus. Virology 15:327-339.

Best, R. J. 1968. Tomato spotted wilt virus. Adv. Virus Res. 13:65-146.

Best, R. R. and Gallus, H. P. C. 1953. Strains of tomato spotted wilt virus. Austr. J. Sci. 15:212-214.

Best, R. J. and Gallus, H.P.C. 1955. Further evidence for the transfer of character determinants (recombination) between strains of tomato spotted wilt virus. Enzymologia 17:207-221.

Best, R. J. and Hariharasubramanian, V. 1967. Serological studies on tomato spotted wilt virus (Strains E and R1). Enzymologia 32:128-134.

Bishop, D. H.L. and Beaty, B. J. 1988. Molecular and biochemical studies of the evolution, infection and transmission of insect bunyaviruses. Phil. Trans R. Soc. Lond. B 321:463-483.

Bishop, D. H. L. and Shope, R. E. 1979. Bunyaviridae. In: Comprehensive Virology, Vol. 14 (ed. H. Fraenkel-Conrat & R. R. Wagner), New York, Plenum Press, pp. 1-156.

Black, L. M., Brakke, M. K. and Vatter, A. E. 1963. Purification and electron microscopy of tomato spotted wilt virus. Virology 20:120-130.

Brittlebank, C. C. 1919. Tomato diseases. J. Agric. Victoria 17:231-235.

Cho, J. J., Mau, R. F. L., Mitchell, W. C., Gonsalves, D. and Yudin, L. S. 1987. Host list of plants susceptible to tomato spotted wilt virus (TSWV). Res. Ext. Series 078, 10 pp.

Cho, J. J., Mau, R. F. L., Hamasaki, R. T. and Gonsalves, D. 1988. Detection of tomato spotted wilt virus in individual thrips by enzyme-linked immunosorbent assay. Phytopathology 78:1348-1352.

de Haan, P., Wagemakers, L., Goldbach, R. and Peters, D. 1989. Tomato spotted wilt virus, a new member of the bunyaviridae. In: Genetics and Pathogenicity of Negative Strand Viruses (Eds. D. Kolakofsky & B.W.J.Mahy). Elsevier Sci. Publ. BV, Amsterdam, pp. 287-290.

de Haan, P., Wagemakers, L., Peters, D. and Goldbach, R. 1990a. The S RNA segment of tomato spotted wilt virus has an ambisense character. J. Gen. Virology 71: 1001-1007.

de Haan, P., Kormelink, R., Peters, D. and Goldbach, R. 1990b. Genetic organization and expression of the tomato spotted wilt virus genome. (These proceedings).

Feldman, J. M. and Boninsega, J. A. 1968. Antiserum to tomato spotted wilt virus. Nature 219:183-184.

Francki, R. I. B. and Grivell, C. J. 1970. An electron microscope study of the distribution of tomato spotted wilt virus in systematically infected Datura stramonium leaves. Virology 42:969-978.

Francki, R. I. B. and Hatta, T. 1981. Tomato spotted wilt virus. In: Handbook of Plant Virus Infections and Comparative Diagnosis (Ed. E. Kurstak), Elsevier/North Holland Biomedical Press: 491-512.

Gonsalves, D. and Trujillo, E. E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.

Huguenot, C., Dobbelsteen, G. van den, de Haan, P., Wagemakers, C. A. M., Drost, G. A., Osterhaus, A.D.M.E. and Peters, D. 1990. Detection of tomato spotted wilt virus using monoclonal antibodies and riboprobes. Arch. Virology 110:47-62.

Ie, T. S. 1970. Tomato spotted wilt virus. CMI/AAB Plant virus Descriptions No. 39.

Ie, T. S. 1971. Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. Virology 43:468-479.

Ie, T. S. 1973. Tomato spotted wilt virus in the anthers of Tropaeolum majus. Neth. J. Plant Pathol. 79:387-391.

Ie, T. S. 1982. A sap-transmissible, defective form of tomato spotted wilt virus. J. Gen. Virology 59:387-391.

Ishikawa, K., Omura, T. and Hibino, H. 1989. Morphological characteristics of rice stripe virus. J. Gen. Virology 70:3465-3468.

Joubert, J. J., Hahn, J. S., von Wechmar, M. B. and van Regenmortel, M.H.V. 1974. Purification and properties of tomato spotted wilt virus. Virology 57:11-19.

Kakutani, T., Hayano, YU., Hayashi, T. and Minobe, Y. 1990. Genome organization of rice stripe virus suggests its phylogenic relationships to phleboviruses (Bunyaviridae). J. Gen. Virology. (In press).

Kitajima, E. W. 1965. Electron microscopy of vira-cabeca virus (Brazilian tomato spotted wilt virus) within the host cell. Virology 26:89-99.

Law, M. D. and Moyer, J. W. 1990. A tomato spotted wilt-like virus with a serologically distinct N protein. J. Gen. Virology 71: 933-938.

Mohamed, N. A., Randles, J. W. and Francki, R.I.B. 1973. Protein composition of tomato spotted wilt virus. Virology 56:12-21.

Matthews, R. E. F. 1982. Classification and nomenclature of viruses. Fourth report of the International Committee on Taxonomy of Viruses. Intervirology 17:1-200.

Milbrath, J. A. 1939. Tomato tip-blight virus. Phytopathology 29:156-168.

Milne, R. G. 1970. An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. J. Gen. Virology 6:267-276.

Milne, R. G. and Francki, R. I. B. 1984. Should tomato spotted wilt virus be considered as a possible member of the family Bunyaviridae. Intervirology 22:76.

Norris, D. O. 1943. Strains of spotted wilt virus and the identity of tomato tip-blight virus with spotted wilt. J. Coun. Sci. Res. Ind. (Aust.) 16:91-92.

Norris, D. O. 1946. The strain complex and symptom development of tomato spotted wilt virus. Counc. Sci. Ind. Res. (Aust.) Bull. 202:55 pp.

Paliwal, Y. C. 1976. Some characteristics of the thrips vector relationship of tomato spotted wilt virus in Canada. Can. J. Bot. 54:402-405.

Ronco, A. E., Dal Bo, E., Ghiringhelli, P. D., Medrano, C., Romanowshi, V., Sarachu, A. N. and Grau, O. 1989. Cloned cDNA probes for the detection of tomato spotted wilt virus. Phytopathology 79:1309-1313.

Samuel, G., Bald, J. G. and Pittmann, H. A. 1930. Investigations on "spotted wilt" of tomatoes. Counc. Sci. Ind. Res. (Aust) Bull. 44.

Tas, P. W. L., Boerjan, M. L. and Peters, D. 1977a. The structural proteins of tomato spotted wilt virus. J. Gen. Virology 36:81-91.

Tas, P. W. L., Boerjan, M. L. and Peters, D. 1977b. Purification and serology of tomato spotted wilt virus. Neth. J. Plant Pathol. 83:61-72.

Tsakirides, J. P. and Gooding, G. V. 1972. Tomato spotted wilt virus in Greece. Phytopathol. Medit. 11:42-47.

Van Kammen, A., Henstra, S. and Ie, T. S. 1966. Morphology of tomato spotted wilt virus. Virology 30:574-577.

Verkleij, F. N. and Peters, D. 1983. Characterization of a defective form of tomato spotted wilt virus. J. Gen. Virology 64:677-686.

Zawirski, I. 1976. Untersuchungen Ueber zwei biologische Typen von Thrips tabaci Lind. (Thysanoptera, Thripidae) in der VR Polen. Arch. Phytopathol. Pflanzenschutz 12:411-422.

THE EFFECT OF HOST SPECIES AND DIFFERENT PLANT COMPONENTS ON THRIPS FEEDING AND DEVELOPMENT

Ronald D. Oetting

ABSTRACT

The western flower thrips, Frankliniella occidentalis (Thysanoptera: Thripidae), has become the major pest on commercial greenhouse production in much of the United States. In experiments on within-plant distribution, western flower thrips were most often found in flowers. If no flowers were present, they were attracted to new plants and the population location depended upon the age of the plant. The developmental time of thrips varies depending upon host species and the part of the host plant they were reared upon. The addition of pollen to the diet increased fecundity and reduces developmental time.

INTRODUCTION

There has been an increase in thrips occurrence on ornamentals over the past decade in the eastern United States and other areas of the world. The most noteworthy of these thrips was the western flower thrips, Frankliniella occidentalis Pergande, which expanded its range from the western states to the east (Oetting, 1986), to mainland Europe (Mantel and van de Vrie, 1988; Bournier and Bournier, 1987) and to other areas of the world (Brodsgaard, 1989). In the 1980's several other thrips species have become more significant pests on crops in the United States: Thrips palmi Karny on vegetables (Johnson, 1986), Echinothrips americanus Pergande on ornamentals (Oetting and Roberts, 1986), Taeniothrips inconsequens (Uzel) on sugar maples (Parker and Skinner, 1988), and other situations have been discussed of other thrips species that were more prevalent in other crops. This increase in thrips significance resulted in increased efforts in the study of thrips and their biology and behavior.

The host range was very broad for many species of thrips even though they may have increased significance on a single crop. The western flower thrips fed on the flowers of many species of plants. Bryan and Smith (1956) reported 139 host records and many plants have been added since then. This species displaced other flower feeding thrips as it expanded its range. These were primarily flower feeding species but they also damage foliage. Other thrips species fed primarily on foliage and also had a large number of host species, e.g. the banded greenhouse thrips on ornamentals (Oetting and Beshear, 1980). Even though these species were classified as foliage feeders, they frequented flowers and probably fed.

The western flower thrips were attracted to plants with flowers. Tests were conducted where flowers of weed species were removed and significantly more thrips were attracted to plants with flowers than without (Yudin et al., 1988). Western flower thrips adults and larvae were more common in flowers than on leaves and stems (Yudin et al., 1986).

Oetting is an associate professor, Department of Entomology, Georgia Agricultural Experiment Station, Georgia, Station, Griffin, GA 30223.

Thrips that lived in flowers fed on pollen, removing the liquid contents and destroying the potential of fertilization (Kirk, 1987). However, they could also possibly aid in pollination by moving pollen grains on their body from flower to flower (Kirk, 1985a). In laboratory rearing, pollen was an important constituent in diets. Frankliniella intonsa Trybom was mass reared on a diet of pollen and honey (Murai and Ishii, 1982). Pollen-feeding could very well play an important part in host selection and susceptibility (Kirk, 1985b). Different cultivars of plants have different susceptibility levels to thrips. These susceptibility levels have been investigated for some thrips, e.g. onion thrips on cabbage (Stoner and Shelton, 1988a,1988b). This variability could be used to select cultivars more resistant to thrips so a more marketable product can be produced with less control measures.

Bryan and Smith (1956) published on the biology of F. occidentalis, including information on larval developmental time. In the last 10 years, several workers have studied the developmental biology of western flower thrips on different hosts. The developmental time varies much with changing temperatures but the host plant also effects the duration, fecundity and egg formation (Brodsgaard, 1989).

The purpose of this paper is to discuss the effect of host plant species on thrips development, different host plant tissue on development, and the distribution of F. occidentalis within a crop of chrysanthemums. Experiments were conducted to determine how the population was distributed upon host plants, both within a single plant and between plants at different stages of development.

FRANKLINIELLA OCCIDENTALIS DISTRIBUTION ON CHRYSANTHEMUMS

Western flower thrips are flower inhabiting thrips but they also feed and oviposit on leaves and other plant tissue. Four experiments were conducted to evaluate the within-plant and between-crop distribution of thrips on greenhouse grown chrysanthemums.

Methods and Materials

Experiments were conducted in two 6.1 X 6.1 M greenhouses. Rooted cuttings of chrysanthemums (Dendranthema grandifolium Tevzel.) cv. 'Manatee Iceberg' were obtained from a commercial propagator and four cuttings each were planted in 15 cm plastic pots. Pots were placed in a propagation area for one week before moving into the growing area. A crop of 25 pots was added to each greenhouse and a crop of mature flowering plants was removed each week. Plant samples were collected each week and were placed in 10 cm diam. Berlese funnels (3 stems each) for thrips extraction. Funnels were covered with a plastic petri dish lid with a hole in the center (covered with silk-screen cloth) for ventilation. A rubber cork was placed on the based of the funnel and a plastic vial containing ethanol placed on the cork to collect thrips. The plant tissue was left in the funnel for 7 days to extract the thrips.

Experiment 1: The first experiment was conducted to study within-plant distribution on plants that were recently moved into the greenhouse and plants that were flowering for one week. A period was selected when populations were low to try to avoid abnormalities resulting from population pressures of high populations. Two areas were collected on the new plants, the terminal section of stem with 2 lower leaves. Four parts of the flowering plants were evaluated: the flower, top 2 leaves, 2 leaves from the middle of the plant, and 2 lower leaves. Four replicates of Three samples were collected from each treatment for a period of 10 weeks.

Experiment 2: This experiment was conducted in greenhouses containing different stages of chrysanthemum growth but no flowers were present. The purpose of the experiment was to observe the distribution of thrips on the terminals of different ages of non-flowering plants. The stages of plant growth sampled were: 4-5 cm plants, 10 cm plants, 25 cm plants (no buds), 25+ cm plants with small buds, and 25+ cm plants with fully developed buds (no color showing). Twenty five terminals (4-5 cm=tip and 2 leaves) of each plant growth stage were sampled. In addition, a section of stem with two leaves was collected from each plant on the plants 10 cm and over.

Experiment 3: An experiment was conducted in greenhouses containing plant of all growth stages to determine where thrips population were the most prevalent. The terminal growing point of different stages of growth were examined. Plant terminals compared were: growing tip, tip with small bud, fully developed bud (no color), bud showing color, newly opened bud with petal vertical, and a newly opened flower with petals fully expanded. Five samples of 3 terminal were collected of each growth stage each week for 10 weeks.

Results and Discussion

Western flower thrips frequented flowers more than other plant parts. However, they were present and fed on all plant parts both as adults and as immatures.

Experiment 1: There were consistently and significantly more thrips in the flowers than other parts of the plant. There was a mean of 57.5 immatures and 10.7 adults per flower. Even though the population of thrips was much higher in flowers there were also immature thrips present on samples of leaves on the upper, middle, and lower stem. The mean number of adults and immatures per leaf was 0.9 and 3.6, respectively. There were no thrips collected on stem samples that did not have leaves.

There were not as many thrips on the young vegetative plants. The population was similar on the leaves and the growing terminal. There were means of 1.1 immatures and 0.8 adults per terminal and 1.5 immatures and 0.4 adults per leaf. These plants had not been in the greenhouse long but thrips had already infested them even though the overall population was low in the greenhouse.

Experiment 2: In experiment 1, there were thrips on the vegetative plants but populations were much lower than found

in flowers. In this experiment there were plants with buds of different maturity but no flowering plants present. The larger buds had the highest population of immature and adult thrips of the terminals samples. There were also more immatures on the lower leaves of these plants than on the plants with smaller buds or only vegetative terminals. However, there was no significant difference in the populations on the different growth stages or location within plants.

Experiment 3: In this experiment only the top of the plant was sampled; flowers, buds, and vegetative growth. The results were consistent with the findings of experiment 1 and the biology of the western flower thrips. They were a flower inhabiting species and most of the population was found in the open flower. The mean population of adults and immatures was 7.7 and 66.4, respectively. The population of thrips per plant terminal increased with maturity until flowering but not significantly, when flowers opened it increased significantly. Flowers were also present in the greenhouse that had been open for a few weeks. If these flowers had been sampled the difference in population levels would be even more significant. The population of thrips per plant terminal increased with maturity until flowering and then it increased significantly.

EFFECT OF DIET ON THRIPS DEVELOPMENT AND OVIPOSITION

The developmental time of the western flower thrips was greatly influenced by temperature but also it varied with different hosts, e.g. at 15°C developmental time, from egg to adult, was 44 days on radish leaves and 34 days on bean pods (Brodsgaard, 1989). The quality of food seemed to have a greater influence on fecundity. Fecundity was much greater when thrips were given whole chrysanthemum flowers rather than radish leaves or bean pods (Brodsgaard, 1989). This increase could have been a result of pollen being present in the chrysanthemum flowers. In an experiment in the Netherlands it was shown that the plant tissue western flower thrips were reared on influenced developmental time (Fransen, personal communication). Thrips developed the fastest on pollen than on leaf tissue and developed the slowest on flower petals of gerbera daisy.

Teulon (1988) studied the effects of pollen and other plant tissue on oviposition and larval development of Thrips obscuratus (Crawford). He found that oviposition could be altered by the presence of pollen. When pollen was supplied daily oviposition was consistent. If no pollen was supplied oviposition was much less. When pollen was present and then removed the oviposition slowed down. If pollen was returned to the diet oviposition resumed at a level comparable to females maintained on pollen. Similar results were obtained by Teulon (1988) for larval development. Larvae developed significantly faster with diets of pollen than on diets of stamen filaments or fruit.

If pollen has an effect on the development and oviposition of thrips, does the type of pollen have any effect on thrips biology? Teulon (1988) investigated the effects of pollen from different species on T. obscuratus. He reared thrips on the pollen of four different host species. Larvae developed on all species with no significant difference in developmental time for males but female development was

significantly faster on one pollen. However, when larvae were supplied only pollen and water, mortality was high. This suggests that thrips require more than just pollen for normal development. In nature pollen would not be found without some other nutritional source but in rearing some other carbohydrate or nutrient source must be provided.

The particular plant tissue that western flower thrips develops upon will affect the duration, especially the addition of pollen. Trichilo and Leigh (1988) studied the life table parameters for F. occidentalis reared on cotton leaves at 27°C. They reported that western flower thrips could develop on cotton leaf tissue alone but the addition of pollen shorten the developmental time. This improved developmental time was especially significant on cotton cultivars which were more resistant to thrips. Trichilo and Leigh also experimented with the addition of mite eggs to cotton leaves. They found that thrips fed upon the mite eggs and it reduced the duration of development. However, pollen was nutritionally better than mite eggs. The addition of pollen lowered the time from egg to adult, increased fecundity, and improved longevity over a diet of only cotton leaves. They hypothesized that when pollen is present, western flower thrips can overcome any negative effects of resistant leaves.

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REFERENCES

Bournier, A. and J.-P. Bournier. 1987. L'introduction en France d'un nouveau ravageur: Frankliniella occidentalis. Phytoma 388: 14-17.

Brodsgaard, H. F. 1989. Frankliniella occidentalis (Thysanoptera; Thripidae)-a new pest in Danish glasshouses. A review. Tidsskr. Planteavl 93: 83-91.

Bryan, D. E. and R. F. Smith. 1956. The Frankliniella occidentalis (Pergande) complex in California (thysanoptera: Thripidae). Univ. Calif. Publ. Entomol. 10: 359-410.

Johnson, M. W. 1986. Population trends of a newly introduced species, Thrips palmi (Thysanoptera: Thripidae), on commercial watermelon plantings in Hawaii. J. Econ. Entomol. 79: 718-720.

Kirk, W. D. J. 1985a. Aggregation and mating of thrips in flowers of Calystegia sepium. Ecol. Entomol. 10: 433-440.

Kirk, W. D. J. 1985b. Pollen-feeding and the host specificity and fecundity of flower thrips (Thysanoptera). Ecol. Entomol. 10: 281-289.

Kirk, W. D. J. 1987. How much pollen can thrips destroy? Ecol. Entomol. 12: 31-40

Mantel, W. P. and M. van de Vrie. 1988. De Californische trips, Frankliniella occidentalis, een nieuwe schadelijke tripssoort in de tuinbouw onder glas in Nederland. Entomol. Ber. Amst. 48: 140-144.

Murai, T. and T. Ishii. 1982. Simple rearing method for flower thrips (Thysanoptera: Thripidae) on pollens. Jap. J. appl. Entomol. Zool. 26: 149-154.

Oetting, R. D. 1986. Biology of thrips on greenhouse crops. Proc. 2nd SAF Conf. on Insect and Disease Management on Ornamentals, Louisville, KY: 28-30.

Oetting, R. D. and R. J. Beshear. 1980. Host selection and control of the banded greenhouse thrips on ornamentals. J. Georgia Entomol. Soc. 15: 475-479.

Oetting, R. D. and J. R. Roberts. 1986. Thrips, a major pest problem on ornamentals. Proc. 31st SNA Res. conf.: 121.

Parker, B. L., M. Skinner, and H. B. Teillon. 1988. Proceedings, Regional Meeting: "The 1988 thrips infestation of sugar maple". Bennington, VT June 23. Vt. Agric. Exper. Sta. Bull. 696, Univ. Vt., Burlington. 113 pp.

Stoner, K. A. and A. M. Shelton. 1988a. Role of nonpreference in the resistance of cabbage varieties to the onion thrips (Thysanoptera: Thripidae). J. Econ. Entomol. 81: 1062-1067.

Stoner, K. A. and A. M. Shelton. 1988b. Influence of variety on abundance and within-plant distribution of onion thrips (Thysanoptera: Thripidae) on cabbage. J. Econ. Entomol. 81: 1190-1195.

Teulon, D. A. J. 1988. Pest management of the New Zealand flower thrips obscuratus (Crawford) (Thysanoptera: Thripidae) on stonefruit in Canterbury, New Zealand. Ph.D. thesis, Lincoln College, University of Canterbury, New Zealand. 221 pp.

Trichilo, P. J. and T. F. Leigh. 1988. Influence of resource quality on the reproductive fitness of flower thrips (Thysanoptera: Thripidae). Ann. Entomol. Soc. Am. 81: 64-70.

Yudin, L. S., J. J. Cho, and W. C. Mitchell. 1986. Host range of western flower thrips, Frankliniella occidentalis (Thysanoptera: Thripidae), with special reference to Leucaena glauca. Environ. Entomol. 15: 1292-1295.

Yudin, L. S., B. E. Tabashnik, J. J. Cho. and W. C. Mitchell. 1988. Colonization of weeds and lettuce by thrips (Thysanoptera: Thripidae). Environ. Entomol. 17: 522-526.

FACTORS AFFECTING THE EPIDEMIOLOGY OF TSWV IN FIELD CROPS: COMPARATIVE VIRUS ACQUISITION EFFICIENCY OF VECTORS AND SUITABILITY OF ALTERNATE HOSTS TO FRANKLINIELLA OCCIDENTALIS (PERGANDE).

R. F. L. Mau, R. Bautista, J. J. Cho, D. E. Ullman, L. Gusukuma-Minuto, and D. Custer

ABSTRACT

Investigation of the virus acquisition efficiency using ELISA of adult thrips (given virus access as larvae) demonstrated differential efficiencies for TSWV acquisition. Western flower thrips (WFT), Frankliniella occidentalis (Pergande), acquired the virus more efficiently than the common blossom thrips, Frankliniella schultzei (Trybom). TSWV was not trans-stadially passed from larvae to adults in the onion thrips, Thrips tabaci Lindeman, and the melon thrips, Thrips palmi Karny.

Ten weed and five crop hosts of TSWV were evaluated in no-choice tests for WFT development. Comparative growth indices were calculated for each host. WFT larvae failed to develop on statice (Limonium latifolium and Emilia sonchifolia.

In assaying WFT oviposition preferences on flowering and non-flowering stages of 5 hosts, we found different preferences for vegetative and flowering stages.

INTRODUCTION

Tomato spotted wilt virus (TSWV) has an extensive host range of more than 200 plant species from 33 dicotyledonous and five monocotyledonous families (Best 1968; Cho et al. 1986; Cho et al. 1987; Iwaki et al. 1984). During recent vegetable epidemics in Hawaii, weed species were implicated as important alternate hosts of the virus and vector (Cho et al., 1986, 1989; Yudin et al., 1986, 1988).

The virus is vectored by thrips species from 3 genera. They are Frankliniella fusca (Hinds), Frankliniella occidentalis (Pergande), Frankliniella schultzei Trybom, Scirtothrips dorsalis Hood, Thrips setosus Moulton and Thrips tabaci Lindeman (Sakimura 1962, Amin et al., 1981, Iwaki et al., 1984). Recent unpublished work on watermelon in Taiwan (Chen, personal communication) and groundnuts in India (Wightman, personal communication) implicates a seventh species, Thrips palmi Karny as a TSWV vector. With the exception of F. fusca and T. setosus, the remaining four vectors and T. palmi occur in Hawaii.

T. tabaci, first noted in Hawaii in 1915, has been implicated with TSWV epidemics on pineapple, tomato and papaya. Sakimura (1946, 1969) was unable to demonstrate TSWV transmission by the F. shultzei (= F. sulphurea) in Hawaii; however, the thrips was shown to vector laboratory isolates of the virus (Cho et al., 1988).

Since the discovery of the western flower thrips, F. occidentalis, in Hawaii, TSWV epidemics occurred in major lettuce growing areas of the state. Induced losses from TSWV during the 1980's were especially severe, and F. occidentalis was shown to be the major vector (Cho et al., 1987; Yudin et al., 1986, 1988).

T. palmi and S. dorsalis were relatively recent immigrants to Hawaii, having been discovered there in 1982 and 1987, respectively. T. palmi has a wide host range and has become a major pest of cucurbit and certain solanaceous crops. S.

dorsalis has not emerged as a significant pest and was infrequently collected.

In working to develop, implement, and refine a TSWV management program for lettuce, the occurrence of several vector species in an area made it difficult for us to make precise recommendations. Inadequate knowledge about vector-virus and vector-host associations hampered our recommendations relating to control of vectors, control of alternate plant hosts and modification of cultural practices. We needed more information on the role and importance of alternate hosts in field epidemics and the relative importance of different vector species.

When this research was commenced more than a year ago, there was little doubt that F. occidentalis was the most important vector in lettuce. Yudin et al. (1986, 1988) provided some knowledge on the host preferences of adult vectors. He showed that behavioral responses of vectors varied according to the plant species and phenological stages in each of the species.

Since virus acquisition is governed by larval feeding and transmission primarily by adult feeding, we needed to identify and categorize hosts which contributed to spread of TSWV to other plants and those which did not. TSWV hosts which support larval development are considerably more important in disease spread than those which do not. Because of the difficulty in identifying thrips larvae from field samples, biological assays were done in the laboratory. We ran no-choice tests to determine the suitability of the common TSWV hosts based on larval development, and subsequently ran choice tests to study the oviposition preference of F. occidentalis.

From our field surveys of alternate hosts of TSWV and information provided by Yudin (1988), we knew that in addition to F. occidentalis, F. shultzei, T. palmi and T. tabaci also occurred in vegetable growing areas of Maui and Oahu. Their host ranges, which were determined from collection of adults, overlapped, and it was possible that the relative importance of the latter three thrips species to disease spread was greater than implied by their relatively low abundance in lettuce fields.

There was reason to believe that vectors did not acquire and transmit TSWV isolates with equal efficiency. In a review of the literature on TSWV vectors, Sakimura (1962) reported that there was little evidence on vector specificity to different strains of the virus, but that vector specificity was possibly due to host preference and seasonal population behavior of vectors. Later, Paliwal (1975) provided evidence in support of vector efficiency and specificity to TSWV isolates. The tobacco thrips (F. fusca) transmitted two Canadian isolates of TSWV more efficiently than did the western flower thrips (F. occidentalis). Furthermore, he found that the onion thrips (T. tabaci) did not transmit two Canadian isolates of TSWV. Amin et al. (1981) reported different transmission efficiencies of vectors of a groundnut isolate of TSWV. F. schultzei transmitted peanut isolates of TSWV more efficiently than did S. dorsalis.

In this paper, we present a progress report of our research. We provide information about the comparative acquisition of one TSWV isolate by four species of thrips, and about the suitability and preference of certain TSWV hosts by F. occidentalis. A portion of the latter study is part of the dissertation research by the second author.

MATERIALS AND METHODS

Acquisition efficiency of vectors. The virus isolate used in this study was obtained from Arctium lappa L. collected at

Kamuela, Hawaii. The isolate was held in Emilia sonchifolia (L.) DC. by thrips transmission with F. occidentalis and occasional sap transmission.

First instar larvae of F. occidentalis, F. shultzei, T. palmi and T. palmi were given acquisition access on systemically infected E. sonchifolia for 2 days, after which they were transferred to acrylic cages (Tanigoshi and Nishio-Wong 1982) and reared to the adult stage. F. occidentalis and F. shultzei were reared on healthy green bean pods, while T. palmi and T. tabaci were fed with healthy cucumber and cabbage leaves, respectively. Food change was made at two to three day intervals.

After the larvae molted to second instar, randomly collected larvae were assayed for TSWV using the double antibody sandwich ELISA method and polyclonal antiserum as described by Cho et al. (1988). The second instar larvae were assayed to determine if the larvae had fed on TSWV infected tissue and to measure the rates of virus passage to the second larval instar. 7- to 10-days old adults were also assayed to determine the proportion of potentially infective adults and to obtain an estimate of the acquisition efficiency of the different species of thrips. Chebychev's equivalent (mean of healthy thrips + 3s) was used as the positive-negative threshold (Sutula et al. 1986).

F. occidentalis host preference and suitability. Thrips were reared in no-choice tests on healthy leaves of Amaranthus hybridus L. (green amaranth), Brassica campestris L. (cabbage), Emilia sonchifolia (L.) DC. (Flora's paintbrush), Fagopyrum esculentum (buckwheat), Galinsoga quadrimaculata (Raf.) Blake (Peruvian daisy), Limonium latifolium (Sm.) Ktze. (statice), Lycopersicon esculentum Mill. (tomato), Nicandra physalodes (L.) Gaertn. (apple of Peru), Nicotiana tabacum L.(tobacco), Sonchus oleraceus (sow thistle, Tropaeolum majus (nasturtium), and Verbena litoralis (verbena). Buckwheat was used as a non-TSWV-host check, although we found out later that it was a TSWV host. First instar larvae were placed in groups of 5 to 10 on the leaves of each host. Each leaf was enclosed in acrylic sandwich-type cages. Insect development was observed daily, and leaves were changed at 2- to 3- day intervals. The tests were carried out at laboratory temperature of about 25 ^{o}C.

Subsequent tests were made on Arctium lappa L. (burdock, gobo), Datura stramonium L. (jimson weed), Lactuca sativa var. longifolia (romaine lettuce), Malva parviflora L. (cheese weed), and Verbesina encelioides (Cav.) Benth. & Hook. (golden crown-beard) under controlled conditions. Rearing at 15, 20 & 26 ^{o}C was done in 3 environmental chambers. Photophase was kept constant at 12D:12L. A total of 15 cages per host was observed at each temperature.

The suitability of the test leaves was compared based on the duration of thrips development and proportion of thrips which completed development. Growth indices (GI) were computed for each host by dividing the mean percentage of adults produced by the mean developmental time (Khan and Saxena 1985).

The feeding and oviposition preference of the adult thrips was observed in choice tests of the vegetative and flowering stages of romaine lettuce, cheese weed, gobo, jimson weed and golden crown-beard. Potted plants were arranged in a 5 x 5 Latin square design inside a large rectangular cage (4 ft x 2 ft x 2 ft). 200-300 thrips were released and allowed to colonize on the plants for 5 days. 3-4 days later plants were dissected and examined for thrips larvae.

RESULTS AND DISCUSSION

Virus acquisition by larvae of different vectors. The vectors acquired TSWV at different rates. ELISA results of the second instar larvae demonstrated that the first instar larvae had acquired virus from infected tissue, and that TSWV was retained through the molt to the second instar larvae. All healthy checks of each species were negative for TSWV.

In comparing the mean numbers of ELISA-positive larvae, we found that the larvae of all four species acquired and passed the virus through molt to the second larval instar. There were more F. occidentalis and F. shultzei which were ELISA positive than were T. tabaci and T. palmi (61.4% and 72% vs. 45.8%, and 24.2%, respectively). However, the differences were not statistically significant when tested by Kruskal-Wallis non-parametric ANOVA ($P<0.07$).

Since prior work had determined a direct relationship of ELISA positive adults and infectivity (Cho et al. 1988), we likewise used the assay to determine and compare the potential infectivity of the thrips. F. occidentalis acquired and retained the virus through the larval and pupal stadia, and substantially more adults were positive for the virus than were those of the other three species. Forty-four percent of adult F. occidentalis were ELISA positive. In contrast, only 2 percent of F. shultzei and none of T. tabaci and T. palmi were positive. The differences in the percentage of ELISA positive adults were statistically significant when tested by Kruskal-Wallis non-parametric ANOVA ($P<0.0043$).

Although we are proceeding with new acquisition and transmission tests, the present results showed a distinct trend in the TSWV acquisition efficiencies of the thrips species. F. occidentalis was more efficient in acquiring the laboratory TSWV isolate used in this study while the other species were comparatively less efficient.

While it is still early to determine the significance of this work, the results seem to provide new evidence in support of a hypothesis of vector specificity in the acquisition of TSWV isolates. Although T. tabaci did not successfully acquire the virus isolate, our numbers tested were small and there is further work to be done.

Nonetheless, the contrast between Sakimura's (1940, 1963) and Paliwal's (1975) research with T. tabaci was too great to easily explain by other means. Further research with different TSWV isolates is needed before we can decide whether there is a basis for vector specificity in the aquisition and transmission of TSWV isolates. Certainly, if reports that T. palmi vectors Asian isolates of TSWV are proven, our results that T. palmi cannot acquire the Hawaiian TSWV isolate may provide added evidence in support of the specificity hypothesis.

F. occidentalis host preference and suitability.

In evaluating the TSWV hosts, we found a considerable range in host suitability to WFT larval development. Between-plant-species variation in the larva to adult developmental times was low. Larva to adult duration ranged from about 9 to 12 days. In contrast, the percentage of thrips which completed their development was a better measure of host suitability. While buckwheat was quite suitable for WFT larval development, WFT did rather poorly on the other test hosts. 76% of the larvae developed into adults on buckwheat. Sowthistle, green amaranth, and cabbage comprised the second group with 22.7, 22.5, and 17.7 percent of the thrips completing their development on these

hosts, respectively. Nasturtium, Peruvian daisy, and verbena were relatively unsuitable for WFT development. Only 9.2, 3.3, 2.5 percent of the larvae developed into adults, respectively. Flora's paintbrush, and statice leaves were completely unsuitable for WFT development; no WFT completed their development on these hosts.
We computed the growth index (GI) for each species to facilitate between-plant-species comparisons. Using the GI's, buckwheat was the most suitable (GI = 7.9). Cabbage, sow thistle and green amaranth comprised the second group (GI = 3.3, 2.3, 1.9, respectively). Peruvian daisy, verbena, nasturtium, Flora's paintbrush, and statice were in the least suitable host group (GI = 0.3, 0.3, 0.8, 0 and 0, respectively).

Subsequent tests have not been completed, but the preliminary results are presented here. Thrips were reared in no-choice tests at three temperatures (15, 20, and 26 °C) on leaves of A. lappa (burdock), D. stramonium (jimson weed), L. sativa var. longifolia (romaine lettuce), M. parviflora (cheese weed), and V. encelioides (golden crown-beard) to further evaluate the suitability of these TSWV hosts. As might be expected the GI was strongly influenced by rearing temperature and host. Lettuce, jimson weed, and cheese weed leaves were the most suitable for WFT development at 26.6 °C (GI = 5.0, 3.8, and 3.4, respectively). Burdock and golden crown-beard leaves were the least suitable (GI = 1.8 and 1.4, respectively). The same relative order of host suitability was observed at 20 and 15 °C. We also observed that the GI's at 20 °C for lettuce, jimson weed, cheese weed, and burdock were approximately 50% of the values for the same host at 26.6 °C, but this relationship did not hold for golden crown-beard.

We have also partially completed an evaluation of WFT oviposition preferences on flowering and non-flowering stages of these five hosts. Our results suggest that the order of preference for vegetative stages were: lettuce > burdock > cheese weed > jimson weed > golden crown-beard. When flowering stages were assayed, the order of preference changed to cheese weed > golden crown-beard > jimson weed > lettuce. We were unable to induce flowering in burdock.

The results of the no-choice rearing tests suggested that certain weed and other hosts of TSWV may not be as important in perpetuating disease epidemics where F. occidentalis is the primary vector. The vegetative stages of apple of Peru, Flora's paint brush, nasturtium, Peruvian daisy, statice, tomato, tobacco, verbena were relatively unsuitable for F. occidentalis larval development and thus would not contribute much to the field densities of viruliferous adults. Vegetative stages of amaranthus, buckwheat, burdock, cabbage, cheeseweed, golden crown-beard, jimson weed, lettuce and sowthistle were likely to be of greater importance in perpetuating TSWV epidemics. Although not surprising due to the predilection of F. occidentalis for flowers, the potential contribution of viruliferous thrips increased greatly when flowering stages of cheese weed and golden crown-beard were tested in choice tests of ovipositional preference.

REFERENCES CITED

Amin, P. W., D. V. R. Reddy, and A. M. Ghanekar 1981. Transmission of tomato spotted wilt virus, the causal agent of bud necrosis of peanut, by Scirtothrips dorsalis and Frankliniella schultzei. Plant Disease 65: 663-665.

Cho, J. J., R. F. L. Mau, T. L. German, R. W. Hartmann, L. S. Yudin, D. Gonsalves, and R. Provvidenti. 1989. A multidisciplinary approach for tomato spotted wilt virus (TSWV) management in Hawaii. Plant Disease. 73: 375-383.

Cho, J. J., R. F. L. Mau, W. C. Mitchell, D. Gonsalves, and L. Yudin. 1987. Host list of tomato spotted wilt virus (TSWV) susceptible plants. University of Hawaii. College of Tropical Agriculture and Human Resources. Research - Extension Series 078. 12 pp.

Cho, J. J., R. F. L. Mau, R. T. Hamasaki and D. Gonsalves. 1988. Detection of tomato spotted wilt virus in individual thrips by enzyme linked immunosorbent assay. Phytopath. 78: 1348-1352.

Cho, J. J., W. C. Mitchell, R. F. L. Mau and K. Sakimura. 1987. Epidemiology of tomato spotted wilt virus disease on crisphead lettuce in Hawaii. Plant Disease. 71: 505-508.

Cho, J. J., R. F. L. Mau, D. Gonsalves, and W. C. Mitchell. 1986. Reservoir Weed Hosts of Tomato Spotted Wilt Virus. Plant Disease. 70: 1014-1017.

Iwaki, M., Y. Honda, K. Hanada, H. Tochihara, T. Yanaha, K. Hokama, and T. Yokoyama 1984. Silver mottle disease of watermelon caused by tomato spotted wilt virus. Plant Disease 68(11): 1006-1008.

Khan, Z. R. and R. C. Saxena. 1985. Behavior and biology of Nephotettix viriscens (Homoptera: Cicadellidae) on tungro virus-infected rice plants: Epidemiology implications. Environ. Entomol. 14: 297-304.

Paliwal, Y. C. 1975. Some characteristics of the thrips Frankliniella vector relationship of tomato spotted wilt virus in Canada. Can. J. Bot. 54(5-6): 402-405.

Sakimura, K. 1969. A comment on the color forms of Frankliniella schultzei (Thysanoptera: Thripidae) in relation to transmission of the tomato spotted wilt virus. Pacific Insects 11(3-4): 761-762. #

Sakimura, K. 1963. Frankliniella fusca, an additional vector for the tomato spotted wilt virus, with notes on Thrips tabaci, another vector. Phytopath. 53: 412-415. #

Sakimura, K. 1962. The present status of thrips-borne viruses. In K. Maramorosch (ed): Biological transmission of disease agents. New York: Academic Press, pp. 33-40.

Sakimura, K. 1946. Two species of thrips non-vector of the spotted wilt virus. J. Econ. Entomol. 39: 398. #

Sakimura, K. 1940. Evidence for the identity of the yellow-spot virus with the spotted-wilt virus: Experiments with the vector, Thrips tabaci. Phytopath. 30: 281-299. #

Sutula, C. L., J. M. Gillett, S. M. Morrissey and D. C. Ramsdell. Interpreting ELISA data and establishing the positive-negative threshold. Plant Disease 70: 722-726.

Tanigoshi, L. K. and J. Y. Nishio-Wong. 1982. Citrus thrips: biology, ecology, and control. Tech. Bull., U.S.D.A. No. 1668. 17 pp.

Yudin, L. S., J. J. Cho, and W. C. Mitchell. 1986. Host range of western flower thrips, Frankliniella occidentalis (Thysanoptera: Thripidae) with special reference to Leucaena glauca. Environ. Entomol. 15: 1292-1295.

Yudin, L. S., B. E. Tabashnik, J. J. Cho and W. C. Mitchell. 1988. Colonization of weeds and lettuce by thrips (Thysanoptera: Thripidae). Environ. Entomol. 17: 522-526.

FACTORS RELATING TO EPIDEMIOLOGY AND SYMPTOMATOLOGY IN FLORIST'S CHRYSANTHEMUM INFECTED WITH THE TOMATO SPOTTED WILT VIRUS.

W.R. Allen, J.A. Matteoni, and A.B. Broadbent.

ABSTRACT

A wide range in susceptibility to the tomato spotted wilt virus (TSWV) was apparent in tests on 36 cultivars of florist's chrysanthemum inoculated mechanically or by thrips. There was a strong correlation between the incidence of infection in mechanically- and thrips-inoculated cultivars, and between symptom expression and susceptibility to the virus. The correlation was strong between natural virus incidence among cultivars and susceptibility to the TSWV, and the correlations was weak between virus incidence and cultivar preferences of the western flower thrips. Symptoms were variable and consisted of chlorotic spots, foliar necrosis, bronzing, ring and line patterns, stem cankers, internal necrosis, and stunting. Cultivars in the same breeding line tended to have similar levels of susceptibility and symptom expression. Losses in commercial greenhouses were associated with reductions in flower weight, petal number, and number of flowers on sprays. Latent infections among cultivars ranged from 1-100%. Significantly more infection occurred under cool ($21^{o}/16^{o}$ C, day/night) than warm ($24^{o}/18^{o}$ C) post-inoculation conditions for all 18 cultivars tested. TSWV was detected (ELISA) in all tissues of thrips-inoculated cultivars grown to flowering in a commercial greenhouse. Virus was most readily detected in stems and new symptomatic leaves followed by roots, and by leaves with primary lesions only. Roots had a higher incidence of virus than asymptomatic leaves, stems, or flowers.

INTRODUCTION

The tomato spotted wilt disease is becoming an increasingly important factor in the production of florist's chrysanthemum (Dendranthema grandiflora Tzvelev) in North America and abroad (Matteoni, 1988a; Matteoni, 1988b; Matteoni et al., 1989a; Matteoni et al., 1989b). This disease is not new to chrysanthemum (Ainsworth, 1932, 1936; Sanford, 1942), but is receiving renewed attention because of its increasing prevalence which is associated with the rapid and widespread dissemination of the western flower thrips (WFT, Frankliniella occidentalis (Pergande)), one of the most aggressive and efficient vectors of the virus (Allen and Broadbent, 1986; Allen and Matteoni, 1987; Green et al., 1988; Halliwell, 1988; MacDonald et al., 1989; Tehrani et al., 1990). Within the last seven years, this thrips has spread extensively across North America and has become the dominant or only species in greenhouses. This species also is becoming widespread in European greenhouses and elsewhere around the world. Symptoms of the disease in chrysanthemum are not well described (Ainsworth, 1936; Ogilvie, 1935; Smith, 1949), and the disease is easily confused with certain fungal and bacterial diseases (Matteoni et al., 1988). Detection of the virus in chrysanthemum by mechanical inoculation has often been difficult, presumably because of low virus concentration and its variable distribution in plants, and because of virus instability. Visual diagnoses and estimates of the incidence of the TSWV in commercial chrysanthemum crops have been complicated by a lack of knowledge of cultivar

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Research Scientists, Agriculture Canada, Research Station, Vineland Station, Ontario, L0R 2E0. J.A. Matteoni now is employed by Westgro Sales, Inc. 7333 Progress Way, Delta, B.C. V4G 1E7.

responses, the relative levels of latent infections, and the similarity of the symptoms to other diseases. This confusion has been eliminated to a great extent largely by the use of ELISA (Gonsalves and Trujillo, 1986; MacDonald et al., 1988; Matteoni et al., 1989) in conjunction with culturing to detect fungi and bacteria.

This paper presents information from a detailed study on symptom expression in TSWV-infected plants of commercially-important cultivars, and on the susceptibility of cultivars to the virus under test and commercial conditions. This information has been related to cultivar feeding preferences of the WFT. The impact of these and other factors on the epidemiology of the disease in greenhouse are discussed.

MATERIALS AND METHODS

Plant Material.

Culture/virus-indexed plants (Yoder Canada Ltd.) were tested in research greenhouses and growth rooms and a commercial greenhouse. The rooms were maintained at ca. 55% RH and at various tempertures under a mixture of sodium vapor and metal halide lamps (200 umols-^{1}m-^{2}PAR at initial plant height, and a 16-h photoperiod). Prior to testing, some plants of each cultivar were grown under conditions to induce flowering, and some were grown under condtions to maintain the vegetative state. Commercial management practices were used throughout the study. Additional plant material was obtained from a commercial greenhouse where the TSWV-WFT complex was well established. These plants also were produced from culture/virus-indexed stock plant, as were plants that were established in the commercial greenhouse to assess the feeding preferences of the WFT.

Antisera Production and ELISA.

Polyclonal antisera were produced in rabbits with preparations of the TSWV purified from Datura stramonium L. (Matteoni and Allen, 1990). The double-antibody sandwich ELISA was used for all serological tests (Allen and Matteoni, 1990). The cut-off limit for a negative reaction was calculated from the mean of the control absorbance value plus three standard deviations, which equaled a value that represented the upper limit of controls at greater than the 99% confidence level (Kendall et al., 1983). Typical control values for all cultivars had a mean absorbance of 0.07 ($\pm$0.005). Plates were read with a Eurogenetics computerized ELISA reader (Model MPR-A4, Tessenderlo, Belgium).

Inoculation, Observation, and Virus Assay.

Plants were mechanically inoculated with a chrysanthemum isolate of the TSWV cultured in D. stramonium L. Inoculum was triturated in a 5^{o} C buffer consisting of 0.01 M TRIS, 0.01 M sodium sulfite, and 0.1% cysteine hydrochloride, pH 7.8-8.0. Carborundum (500-grit) was dusted on the leaves of test and control plants prior to inoculation, and leaves were rinsed with tap water after inoculation. Control plants were inoculated with the buffer alone. Inoculations were done twice: 1 and 3 weeks after potting. Nicotiana glutinosa L. was included in each inoculation to assess the potency of the inoculum. One of each chrysantemum cultivar was included in each set to be inoculated, and fresh inoculum was prepared for successive sets to ensure that inoculum potency remained high during inoculation. To account for any reduction in potency of the inoculum, the order of inoculation for the cultivars in each successive set was randomized. Serological assays were conducted on a portion (ca. 0.5 g) of each of 4 inoculated leaves/plant 2 weeks after inoculation. At 8 weeks post-inoculation, test were repeated on tissues from 2 inoculated and 2 noninocu-

lated leaves. Assays were repeated once on negative plants by testing 2 additional noninoculated leaves.

Assays to assess the incidence of virus in cultivars grown under commercial conditions were done on a single leaf/plant selected on the basis of presence of symptoms or thrips feeding scars. The first 10 plants in each of ca. 10 transects/cultivar were sampled. In total, 22 cultivars and over 1800 plants were tested. ELISA also was done on all tissues of at least 10 infected plants of each cultivar grown to flowering in the commercial greenhouse, in order to determine virus distribution within the plants.

Cultivar Feeding Preferences of the WFT.

Repeated and randomized tests with 27 cultivars were conducted in a growth room programmed to give $25^{o}/22^{o}$ C, day/night, 75% RH, and 180 umols-^{1}m-^{2}PAR of mixed fluorescent and incandescent light at plant height for a 16-h photoperiod. Apparent feeding preference was assessed after 2 weeks by counting feeding scars (> 1 mm) on 1 pre-selected middle leaf of each of 5 plants/ cultivar. Scarring also was subjectively evaluated by ranking those leaves according to the leaf area damaged. The leaves were attached to cards, and the cards were ordered from least to most damaged and ranked numerically (Broadbent et al., 1990).

Statistical Analyses.

Tranformed data were analyzed and compared using Student's t-test, ANOVA, LSD, Duncan's Multiple Range Test, and regression where appropriate. Both SAS and StatView II software were used for the analyses (Broadbent et al., 1990; Matteoni and Allen, 1990).

RESULTS

Symptomatology.

Symptoms induced by the TSWV in chrysanthemum cultivars included chlorotic spots or ring patterns in inoculated leaves; chlorotic spots, ring patterns, bronzing or foliar necrosis in new leaves; and stem distortion or stem cankers and stunting. Not all symptoms occurred in all cultivars (Matteoni and Allen, 1990).

The most common symptoms were local chlorotic spots in inoculated leaves, and foliar necrosis and chlorotic spotting in new leaves. Similar local chlorotic spotting also developed around feeding scars when the virus was transmitted by thrips. Cultivars in the Super, Marble, and Chardonnay (Charlie, Charisma, and El Charo) lines were almost entirely free of ringspots, line patterns, stem cankers, and stunting, both in the growth room and in commercial greenhouses. These symptoms were associated with significantly ($P<0.001$) fewer plants of all cultivars than the aforementioned leaf symptoms.

Symptoms resulting from mechanical inoculation were similar in all respects to those observed in thrips-inoculated plants in commercial greenhouses. There were no differences in symptom expression in vegetative or flowering plants of each cultivar.

Chlorotic spots developed in inoculated leaves in 10-14 days; all other symptoms required at least 4 weeks to develop. Stem cankers required 6-8 weeks to develop, and were frequently adjacent to or above inoculated leaves with chlorotic spots. Inoculated plants which were maintained vegetatively tended to outgrow symptoms in about 6 weeks, although they remained positive by ELISA for at least 26 weeks after inoculation.

Effect of the TSWV on Flower Production.

The fresh weight of flowers of 7 infected cultivars from a commercial greenhouse was significantly ($P<0.01$ to $P<0.001$) reduced (range, 13-51%) (Fig. 1). The number of petals also was significantly reduced ($P<0.025$ to $P<0.001$) on 3 of the 7 cultivars (range, 9-19%) (Fig. 2). Further, the number of open flowers per stem at the time of cutting was reduced significantly ($P<0.025$ to $P<0.001$) on 6 of the 7 cultivars (range, 15-35%) (Fig. 3). Also, infected plants of some cultivars flowered as much as 1.5 weeks later than adjacent healthy plants (Matteoni and Allen, 1990).

Susceptibility of Cultivars to Mechanical Inoculation.

A wide range in the incidence of the TSWV among culitvars was apparent after mechanical inoculation, as assessed by ELISA (Fig. 4) (Allen and Matteoni, 1990). Overall, the incidence of infection in vegetative or flowering plants was not significantly different ($P=0.05$). A mean of 50% (range, 8-85%) of the plants of all cultivars were infected, and there was a strong tendency for sister cultivars to have similar incidences of infection; e.g. Palisade and Yellow Palisade (Fig. 5). A comparison of yellow- and white-flowered cultivars did not indicate a significance difference ($P=0.05$) in susceptibility, nor were there differences in symptom expression.

In general, the incidence of economically important symptoms (i.e., stunt, foliar necrosis, and canker) was higher in the more susceptible cultivars; such as, the Palisades, Polaris', May Shoesmiths, and Gold- and Icecap (Fig. 6). Overall, there was a significant correlation ($R^2=0.61$, Adj. $R^2=0.59$, $P=0.0001$) between the incidence of TSWV and symptoms expression among cultivars.

Latent infections occurred in all 36 cultivars tested, and latency at levels of 50% or higher occurred in 35% of the cultivars (Fig. 7). For cultivars with 50% or higher incidences of infection, latency ranged from 1-58% (mean, 26%). Overall, the highest incidences of latent infection (range, 74-100%) occurred in the less susceptible cultivars; e.g. Super Yellow, Super White, Iceberg, Amber, and Omegon. Cultivars within the same breeding line (e.g. Palisades, Polaris', and Marbles) had similar levels of latency with some exceptions.

Influence of Temperature on Susceptibility.

The proportion (61%) of infected plants under a post-inoculation cool temperature regime ($21^{o}/16^{o}$ C, day/night) was significantly greater ($P<0.0001$) than the proportion (23%) under the warm regime ($24^{o}/18^{o}$ C, day/night) (Fig. 8). However, there was no significant difference ($P=0.05$) between the proportion of infected plants which developed symptoms under the cool (54%) adn warm (57%) regimes. The greatest differences in infection between the two regimes were apparent in the Dynamo line and Charlie, followed by Yellow Polaris, El Charo, and Blue Marble. The differences ranged from 55-80%. Overall, the changes in virus incidence among cultivars from warm to cool regimes ranged from 5 to 80% (mean, 38%). The slightly higher incidences of latent infection under the cool regime was not significant ($P=0.05$).

Incidence of the TSWV in Naturally-Inoculated Cultivars.

Studies in a commercial greenhouse included 17 of the cultivars tested by mechanical inoculation. As in the latter test, there was a wide range in the incidence of the TSWV among cultivars. Also, the incidence of virus was similar in sister cultivars. The greatest range in virus incidence occurred among cultivars in the Standard and

Decorative groups. Overall, there was a significant correlation (R^2=0.75, Adj. R^2=0.73, P=0.001) between the natural incidence of the virus in these cultivars and their relative susceptibility to mechanical inoculation, indicating that mechanical inoculation trials are useful in predicting "field performance." Latent infections occurred in all cultivars and ranged from 2-100% (mean, 43%). Latency at levels of 50% or more occurred in 32% of the cultivars. For cultivars with incidences of infection of 50% or higher, latency ranged from 2-87% (mean, 27%). Overall, the highest incidences of latent infection (range, 75-100%) occurred in the less susceptible cultivars; e.g., Supers, Jet Set, and Omegon. These results agreed closely with those from the mechanical-inoculation tests.

Distribution of the TSWV in Tissues of Selected Cultivars.

TSWV was detected by ELISA in all tissues of 14 cultivars that were naturally infected and grown to flowering in a commercial greenhouse (Figs. 9,10). The virus was most readily detected in symptomatic leaves and stems, followed by roots, and leaves with primary symptoms only. Roots, with some exceptions, had a higher incidence of virus than asymptomatic leaves, stems, or flowers. Overall, the mean range in virus detection for all tissues and cultivars was 76%. The percentage of virus incidence among mechanically-inoculated cultivars was significantly correlated (R^2=0.66, Adj. R^2=0.63, P<0.001) with the mean percent of virus detection in all tissues of the same cultivars.

Cultivar Preferences of the WFT.

Based on feeding scar counts and ranking according to leaf area damaged, there was a wide range in cultivar preference of the WFT among 27 cultivars tested under growth room conditions (Fig. 11) (Broadbent et al., 1990). The widest range occurred in the Daisy group which contained the highly preferred Marble cultivars. Similar differences in cultivar preference occurred under commercial conditions when the white-flowered cultivars White Marble, Polaris, Super White, and Chardonnay were established randomly in blocks at the ends of production beds (Broadbent et al., 1990).

A comparison of scar numbers on leaves of white- and yellow-flowered sister cultivars (e.g. Polaris and Yellow Polaris) indicated a significant (P<0.01) preference for yellow-flowered cultivars, even though the plants were in the pre-bloom stage (Fig. 12).

There was a weak linear correlation (R^2=0.30, Adj.R^2=0.25, P=0.029) and a strong nonlinear correlation (R^2=0.67, Adj. R^2=0.62, P=0.0007) (Fig. 13) between cultivar preference and virus incidence among cultivars grown in the commercial greenhouse. The weak correlation was created by 4 of the 16 cultivars being compared. Those few cultivars (viz., White and Blue Marble and the Dynamos) were highly preferred by the WFT, but were only poorly to moderately susceptible to the virus (Fig. 7). Consequently, natural virus incidence was low. When the 4 cultivars were excluded from the analysis, a strong linear correlation (R^2=0.95, Adj.R^2=0.95, P=0.0001) was indicated, suggesting that thrips preference and virus incidence were closely related for the majority of the cultivars.

DISCUSSION

Infection of chrysanthemum cultivars by the TSWV resulted in a variety of symptoms of which chlorotic spotting and foliar necrosis predominated. Other symptoms which could be of considerable economic importance included stem cankers and decreased flower quality. Ringspots, although diagnostic for the virus disease (Anonymous, 1952; Matteoni and Allen, 1990), were not common. Previous reports on symptoms

induced by the virus in chrysanthemum are limited, but such symptoms included vein clearing (Bald and Samuel, 1931); chlorotic blotches and ringspots (Anonymous, 1949; Anonymous, 1952; Smith, 1935); and stunting, metallic bronzing, and brown spotting of leaves and stems (Ainsworth, 1932; Smith, 1935). The primary differences in symptomatology in the cultivars we tested were the absence of vein clearing and the extensive occurrence of stem lesions and internal stem discoloration on some cultivars (Matteoni and Allen, 1990).

The effects of the TSWV on flower quality are not well documented (Anonymous, 1947) and were more dramatic than expected. Reductions in fresh weight, and petal number and size, resulted in reduced grade, but these effects were usually not as important as the substantial reductions in flower numbers on sprays.

As with other hosts of the TSWV, the response to infection differed considerably among cultivars (Anonymous, 1949, 1952). There was an obvious tendency for cultivars in the same breeding line to respond similarly to infection. This was noteworthy in the Marble and Super lines for which stem canker and stunting were not noted. In contrast, these symptoms were prevalent in the Palisade, Ice- and Goldcap, and Polaris lines. This information and the results from the susceptiblity tests clearly indicate that growers can reduce losses where inoculum pressure is high by substituting resistant cultivars from different breeding lines having similar flower characteristics. Similarly, breeders can make use of the information to develop resistant cultivars.

Overall, symptom expression among culitvars correlated positively with susceptibility to the virus, as measured by the incidence of infection after mechanical or natural inoculation by thrips. Further, there was a strong correlation between the natural incidence of the virus and the incidence induced by mechanical inoculation, indicating that mechanical inoculation tests are useful in predicting cultivar responses under commercial conditions.

Temperature effects on the tomato wilt disease have been noted for florist's chrysanthemum (Gardner et al., 1935), tomato (Gardner and Whipple, 1934)), and cyclamen (Allen, Matteoni, 1988), and the present study indicated that the effect also is significant on currently-grown chrysanthemum cultivars. Since some cultivars may be grown the year round, it is important to assess new or untested cultivars under summer and winter temperature conditions to evaluate their response to infection. Our results from growth room tests agreed with observations made seasonally over several years in commercial greenhouses. Experience indicates that susceptible cultivars should not be grown under cool conditions if the virus-thrips complex occurred at significant levels in the preceeding crop. Even when populations densities of the thrips are low, significant losses can occur under cool conditions due to often dramatic increases in both susceptibility to the virus and disease severity. The high levels of latent infection among cultivars appears to explain why roguing practices by growers have had little effect in reducing sources of inoculum and the consequently high incidences of infection in susceptible cultivars. The low levels of symptom expression in certain cultivars has led some growers to falsely assume that these cultivars are more resistant to the virus than our tests indicate, and that stock plants of these cultivars can be safely maintained, thus avoiding the cost of new material. Consequently, these cultivars have become extensively infected and have served as continuing sources of inoculum for virus-free stock obtained from commercial propagators. Because of these poor management practices, propagators are often unjustly blamed for distributing infected stock. Moreover, another factor contributing to rapid virus

dissemination in some greenhouses is the practice of situating rooting benches and stock beds adjacent to production beds. Even when virus-free cutting are used, the incidence of the virus can develop to significant levels by the time cuttings are ready for planting or when stock plants are large enough to provide cuttings. Eradication of the virus has been possible only when a complete break in cropping was implemented.

Ideally, growers concerned with controlling this virus-thrips complex should use cultivars that are unattractive to the thrips and resistant to the virus. However, few cultivars meet both requirements; only Accent, Super Yellow, and Super White were identified in these studies (Fig. 14, Group 1). Moreover, the best alternatives would be the use of poorly to moderately virus-susceptible cultivars that are poorly to moderately attractive to thrips; e.g. Icebergs, Chardonnay, and Omegon (Fig. 14, Groups 2 and 3). Generally, these cultivars have demonstrated satisfactory levels of field resistance. Cultivars that are resistant to infection, but are moderately to highly attractive to thrips, also generally have acceptible levels of field resistance; e.g. White Marble and Amber (Fig. 14, Group 4). Therefore, the overall results appear to indicate that the virus susceptibility of cultivars is of primary consideration in controlling the disease, and that differences in cultivar preference by the WFT influence disease incidence most with moderately susceptible cultivars and least with poorly and highly susceptible cultivars. These relationships are exemplified (Fig. 15) by White Marble which is poorly susceptible to the virus, highly preferred by the thrips, and has a relatively low natural incidence of the virus; by Dynamo which is moderately susceptible to the virus, somewhat less preferred by the thrips than White Marble, but has a higher natural incidence of the virus than White Marble; and by Palisade which is highly susceptible to the virus, less preferred by the thrips than Dynamo, and has the highest natural incidence of the virus. Moreover, these results apparently are not in agreement with those of Amin and Singh (Reddy and Wightman, 1988) who reported that cultivar preference of F. schultzei (Trybom) was the overriding factor in determining field susceptibility to the TSWV in peanut.

Whereas, management of the TSWV-WFT complex must take into consideration cultivar susceptibility to the virus and latency, economic impact of the disease, and cultivar preferences of the WFT, another important factor must be considered; namely, the reproductive rate of the WFT on these cultivars. Cultivars that are preferred for egg laying are undesirable even though they may be virus resistant, since they constribute to high vector populations. This aspect of the management scheme is under study.

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REFERENCES

Ainsworth, G.D. 1932. Virus disease investigations. Page 39 in: Cheshunt Exp. Res. Sta. 18th Ann. Rept., 1939.

Ainsworth, G.D. 1936. Detection of spotted wilt virus in chrysanthemums. Nature 137:868.

Allen, W.R. and A.B. Broadbent. 1986. Transmission of tomato spotted wilt virus in Ontario greenhouses by Frankliniella occidentalis. Can. J. Plant Pathol. 8:33-38.

Allen, W.R. and J.A. Matteoni. 1988. Cyclamen ringspot: Epidemics in Ontario greenhouses caused by the tomato spotted wilt virus. Can. J. Plant Pathol. 10:41-46.

Allen, W.R., J.A. Matteoni, and A.B. Broadbent. 1990. Susceptibility of cultivars of florsit's chrysanthemum to the tomato psotted wilt virus. Can. J. Plant Pathol. (in press).

Anonymous. 1947. Diseases of chrysanthemums. Agric. Gaz. N.S.W. 58:535-538.

Anonymous. 1949. Spotted wilt of ornamental plants. J. Agric. Victoria. 47:230-232.

Anonymous. 1952. Chrysanthemums. Bull. Minist. Agric. Lond. #92. 50 pp.

Bald, J.G. and G. Samuel. 1931. Investigations on 'spotted wilt' of tomatoes. II. Austr. Counc. Sci. & Indus. Res. Bull. 54. 24 pp.

Broadbent, A.B., W.R. Allen, and R.G. Footitt. 1987. The association of Frankliniella occidentalis (Pergande) (Thysanoptera:Thripidae) with greenhouse crops and the tomato spotted wilt virus in Ontario. Can. Ent. 119:501-503.

Broadbent, A.B., J.A. Matteoni, and W.R. Allen. 1990. Feeding preferences of the western flower thrips, Frankliniella occidentalis (Pergande)(Thysanoptera: Thripidae) for cultivars of florist's chrysanthemum. Can. Ent. (in press).

Gardner, M.W., C.M. Tompkins, and O.C. Whipple. 1935. Spotted wilt of truck crops and ornamental plants. Phytopathology 25:17.

Gardner, M.W. and O.C. Whipple. 1934. Spotted wilt of tomato and its transmission by thrips. Phytopathology 24:17.

Gonsalves, D. and E.E. Trujillo. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.

Green, J.L., T.C. Allen, and S. Fischer. 1988. Tomato spotted wilt virus in Stephanotis in an Oregon greenhouse. Plant Dis. 72:912.

Halliwell, R.S. 1988. Tomato spotted wilt virus infection of cultivated malvaceous species and other crop plants in south Texas, U.S.A. Phytopathology 69:87-88.

Kendall, C., I. Ironescu-Matiu, and G.R. Dreesman. 1983. Utilization of the biotin/avidin system to amplify the sensitivity of the enzyme-linked immunosorbent assay (ELISA). J. Immunol. Meth. 56:329-339.

MacDonald, L.S., R. Stace-Smith, W.R. Allen, and J.A. Matteoni. 1989. Incidence of tomato spotted wilt virus in British Columbia. Can. Plant Dis. Surv. 68:87-88.

Matteoni, J.A. 1988a. Tomato spotted wilt virus and thrips: Growing concerns for floriculture. Pages 104-105 in: Proceedings Fourth Conference on Insect and Disease Management on Ornamentals. Soc. Amer. Florists. Alexandria, Va. 214 pp.

Matteoni, J.A. 1988b. Disease of florist's chrysanthemum in Ontario from 1983 to 1987. Can. Plant Dis. Surv. 68:85-86.

Matteoni, J.A. and W.R. Allen. 1990. Symptomatology of tomato spotted wilt virus infection in florist's chrysanthemum. Can. J. Plant Pathol. 11: 373-380.

Matteoni, J.A., W.R. Allen, and A.B. Broadbent. 1988. Host range and symptoms of tomato spotted witl virus. Pages 84-93 in: Proceedings Fourth Conference on Insect and Disease Management on Ornamentals. Soc. Amer. Florists, Alexandria, Va. 214 pp.

Matteoni, J.A., W.R. Allen, and A.B. Broadbent. 1989a. Tomato spotted wilt virus in greenhouse crops in Ontario. Plant Dis. 72:801.

Matteoni, J.A., W.R. Allen, and A.B Broadbent. 1990b. Susceptibility of florist's chrysanthemum to tomato spotted wilt virus and western flower thrips. Phytopathology 79:1149.

Matteoni, J.A., W.R. Allen, and B. Tehrani. 1989c. Diseases of ornamentals: Diagnostic summary, Vineland Research Station. Can. Plant Dis. Surv. 69:90-94.

Ogilvie, L. 1935. Spotted wilt of tomatoes and its control. Pages 170-174 in: Annu. Rept. Agric. Hortic. Res. Sta. Bristol., 1934.

Reddy, D.V.R. and J.A. Wightman. 1988. Tomato spotted wilt virus: Thrips transmission and control. Pages 203-220 in K.F. Harris, ed. Adv. Disease Vector Research. Vol. 5. Springer-Verlag. New York.

Sanford, G.B. 1942. Incidence of viruses in ornamentals. Can. Plant Dis. Surv. 21st Annu. Rept. 89 pp.

Smith, K.M. 1935. Some diseases of ornamental plants caused by the virus of tomato spotted wilt. J. R. Hortic. soc. 60:304-310.

Smith, K.M. 1949. Viruses and virus diseases. Part II. J.R. Hortic. Soc. 74:521-528.

Tehrani, B., J.A. Matteoni, and W.R. Allen. 1990. Update on the incidence of tomato spotted wilt virus in Canadian greenhouses. Can. Pl. Dis. Surv. 70:(in press).

APPENDIX

Cultivar Characteristics and Abbreviations Used in Figures.

Cultivar	Flower color	Group	Abbreviation
Accent	Pink	Daisy	AC
Amber	Bronze	Daisy	AM
Blue Marble	Pink	Daisy	BM
Chardonnay	White	Daisy	CD
Charlie	Yellow	Daisy	CH
Charisma	Pink	Daisy	CR
Dynamo	White	Decorative	D
El Charo	Bronze	Daisy	EC
Foxy	Red	Daisy	F(FX)
Florida Marble	Yellow	Daisy	FM
Goldburst Mefo	Yellow	Standard	GM
Goldcap	Yellow	Pompom	GC
Golden Polaris	Yellow	Decorative	GP
Goldflo	Yellow	Pompom	GF
Iceberg	White	Decorative	I
Icecap	White	Pompom	IC
Iceflo	White	Pompom	IF
Improved Mefo	White	Standard	IM
Jet Set	Pink	Daisy	JS
May Shoesmith	White	Standard	MS
Maximo	White	Decorative	M(MX)
Mellow	Yellow	Daisy	ML
Omegon	White	Standard	O
Palisade	White	Standard	PS
Polaris	White	Decorative	P
Super White	White	Spider	SW
Super Yellow	Yellow	Spider	SY
Vero	White	Daisy	V
Westland Snow	White	Spider	WSW
Westland Sun	Yellow	Spider	WSN
White Delight	White	Decorative	WD
White Marble	White	Daisy	WM
Yellow Dynamo	Yellow	Decorative	YD
(Dark) Yellow Iceberg	Yellow	Decorative	YI
(Bright) Yellow May Shoesmith	Yellow	Standard	YMS
Yellow Palisade	Yellow	Standard	YPS
Yellow Polaris	Yellow	Decorative	YP

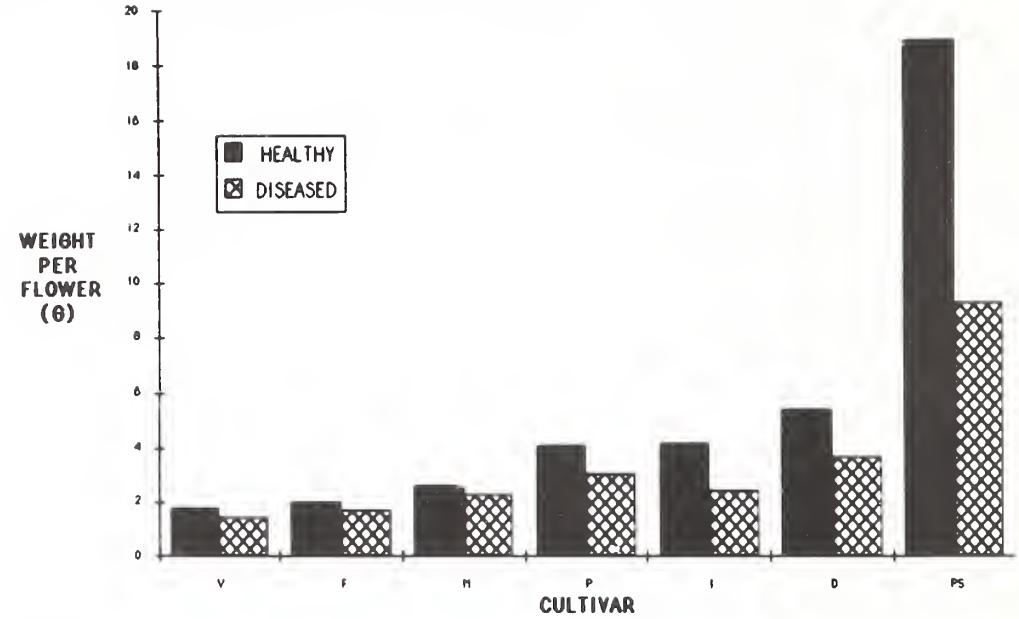

Figure 1. Influence of the TSWV on flower weight among chrysanthemum cultivars (see APPENDIX for cultivar names).

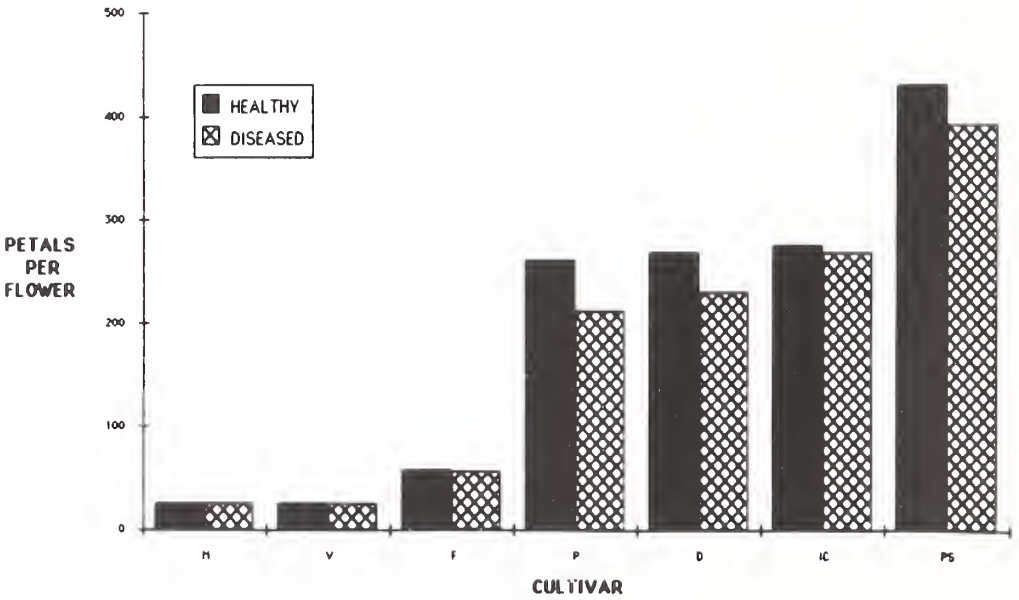

Figure 2. Influence of the TSWV on flower petal number among chrysanthemum cultivars (See APPENDIX for cultivar names).

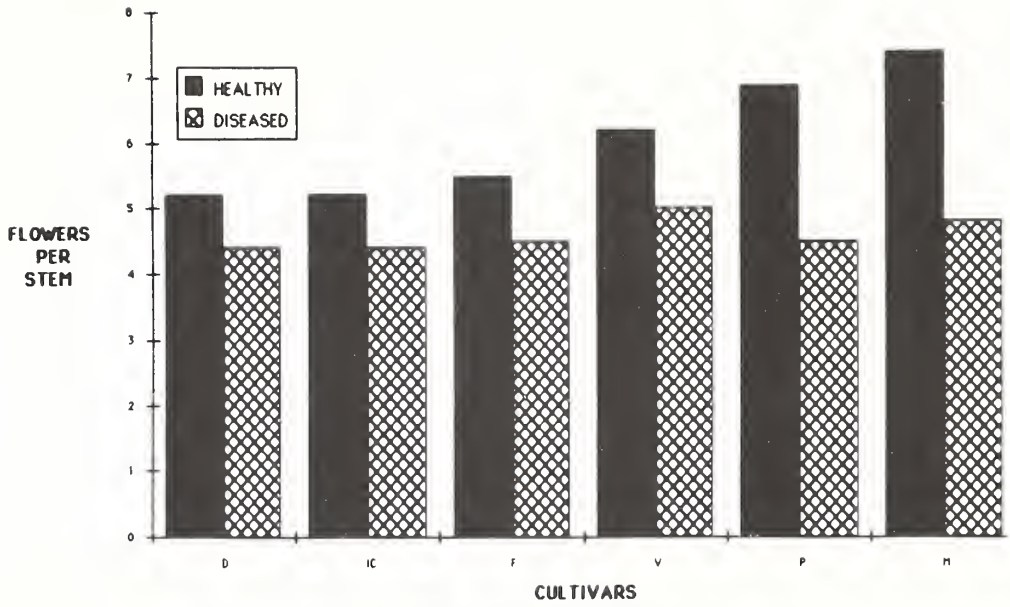

Figure 3. Influence of the TSWV on flower number among chrysanthemum cultivars (See APPENDIX for cultivar names).

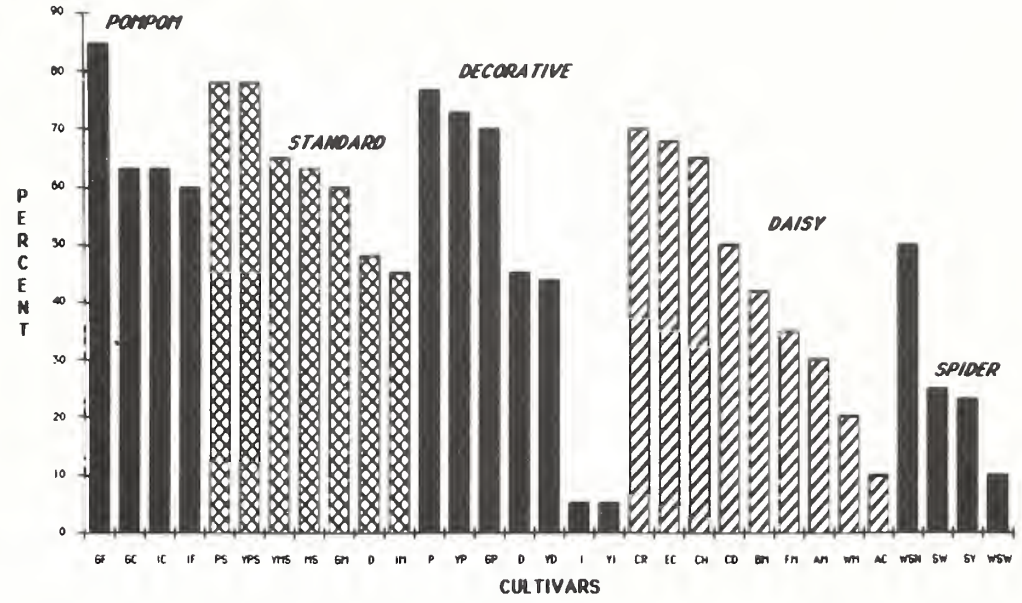

Figure 4. Relative susceptibilities (% infected) of chrysanthemum cultivars to mechanical inoculation with the TSWV (See APPENDIX for cultivar names.

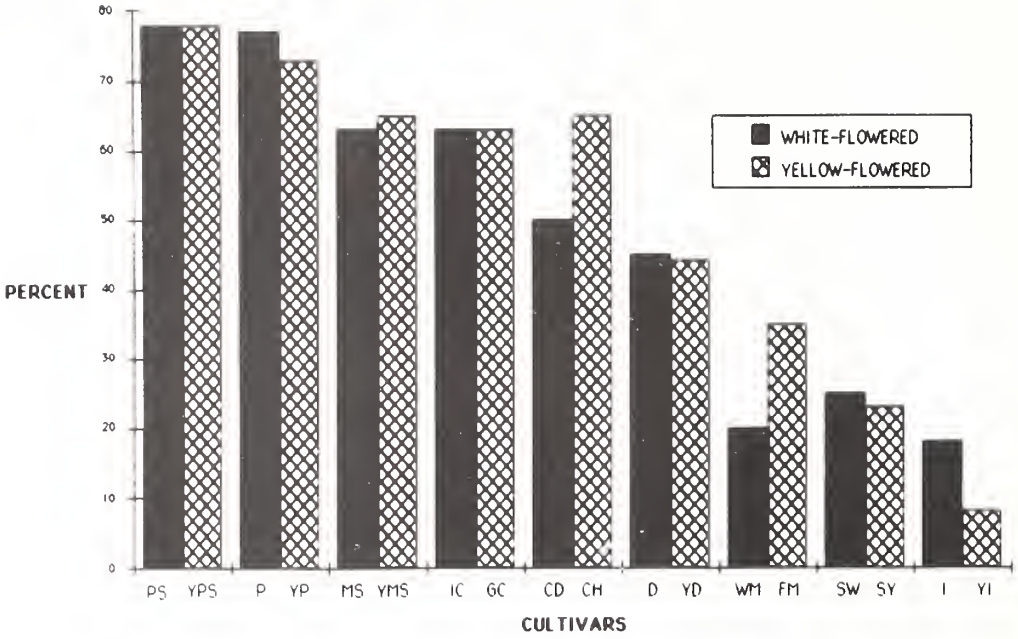

Figure 5. Relative susceptibilities (% infected) of white- and yellow-flowered sister cultivars to mechanical inoculation with the TSWV (see APPENDIX for cultivar names).

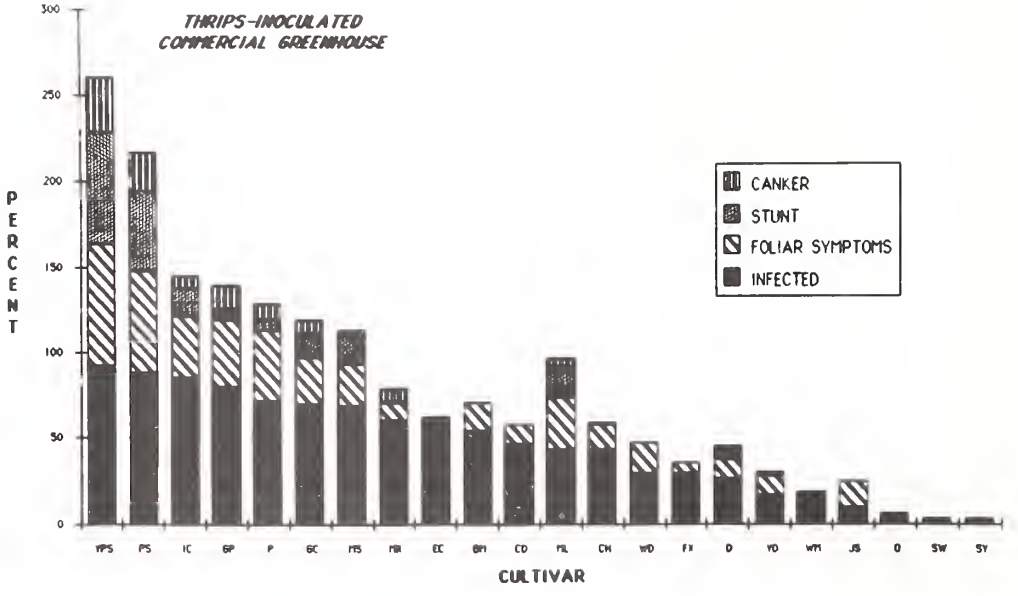

Figure 6. The relation between susceptibility (% infected) to the TSWV and symptom expression (see APPENDIX for cultivar names).

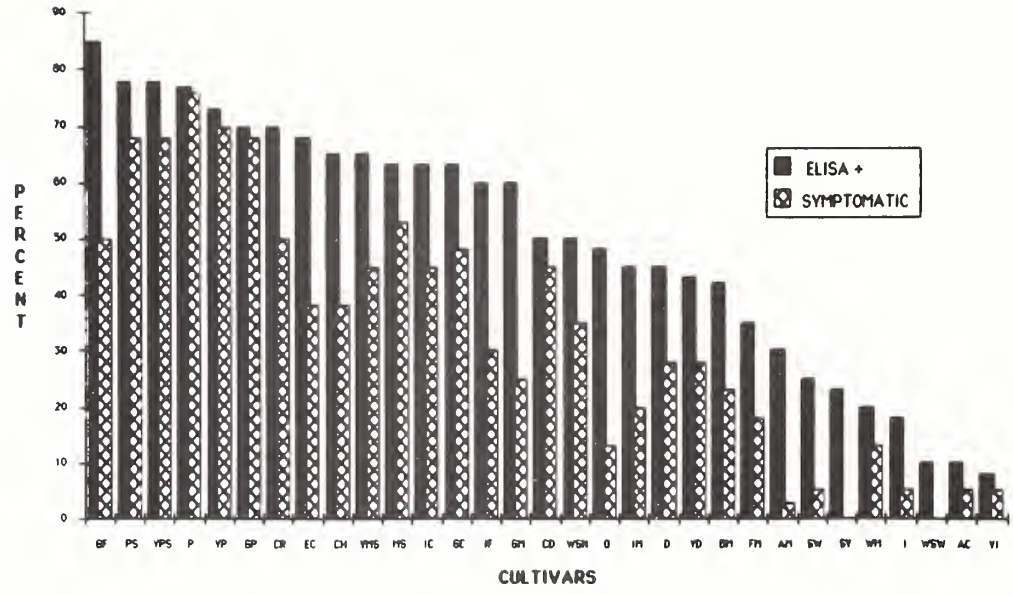

Figure 7. Relative latency of the TSWV infections in chrysanthemum cultivars (See APPENDIX for cultivar names).

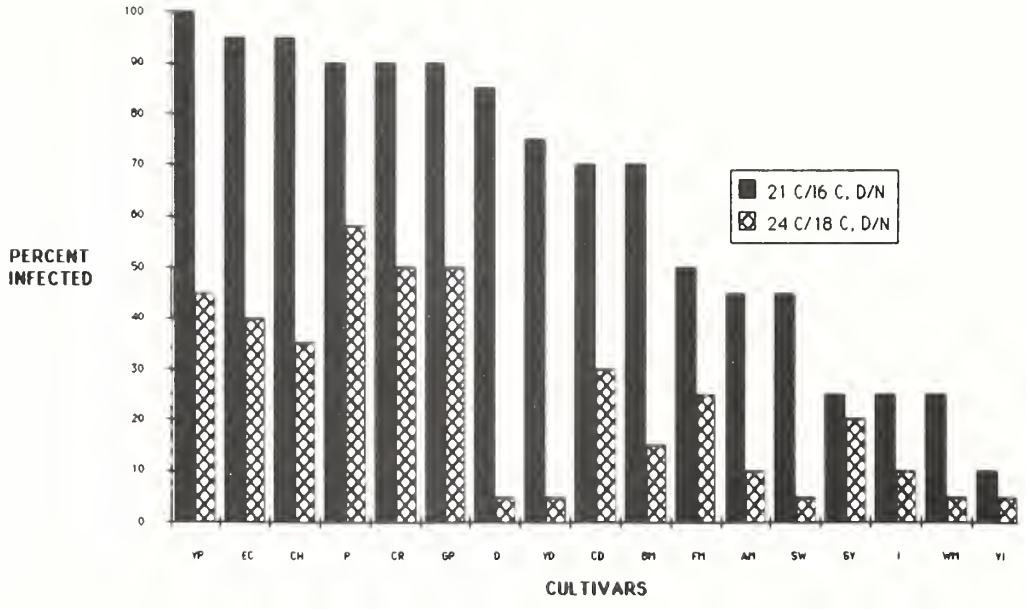

Figure 8. Influence of post-inoculation temperature regimes on infection of chrysanthemum cultivars by the TSWV (See APPENDIX for cultivar names).

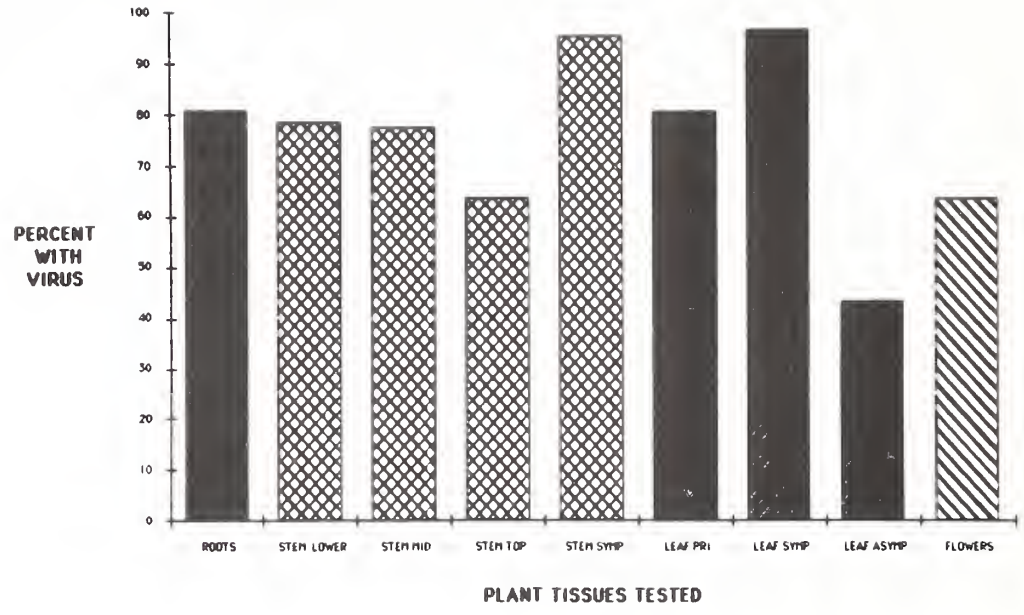

Figure 9. Mean distribution of the TSWV among tissues of 14 chrysanthemum cultivars grown to flowering in a commercial greenhouse. (Symp.=symptomatic; asymp.=asymptomatic; pri=primary).

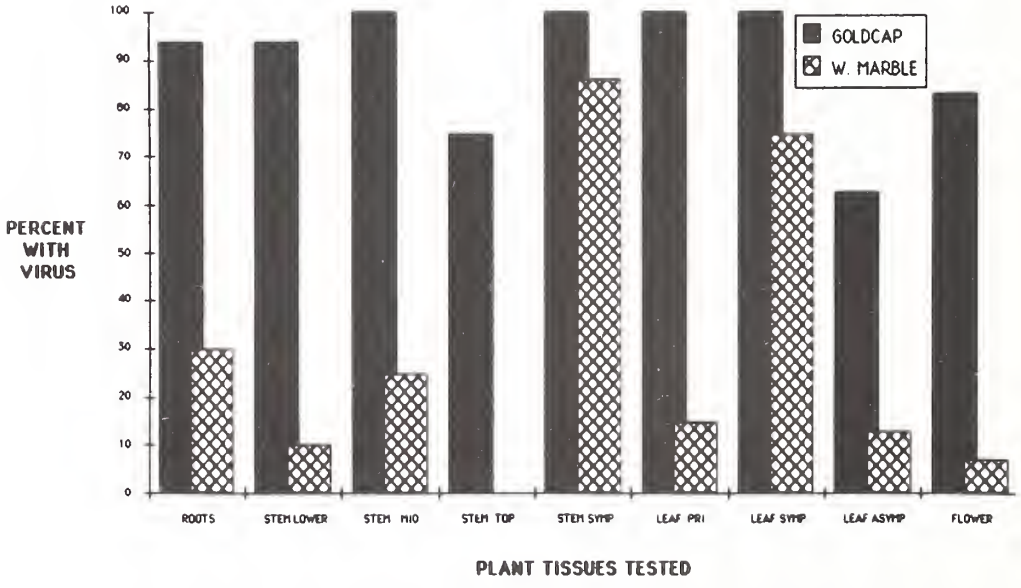

Figure 10. Relative distribution of the TSWV among tissues of susceptible (cv. Goldcap) and resistant (cv. White Marble) chrysanthemum cultivars grown to flowering in a commercial greenhouse. (Symp.=symptomatic; asymp.=asymptomatic; pri=primary).

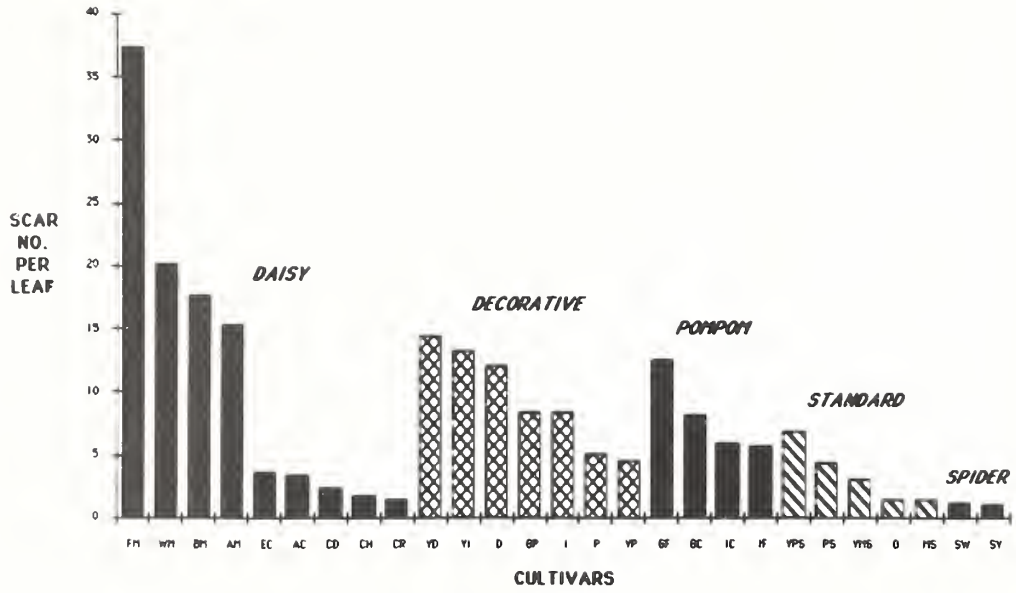

Figure 11. Feeding preferences of the WFT among 27 chrysanthemum cultivars, based on feeding scar counts (See APPENDIX for cultivar names).

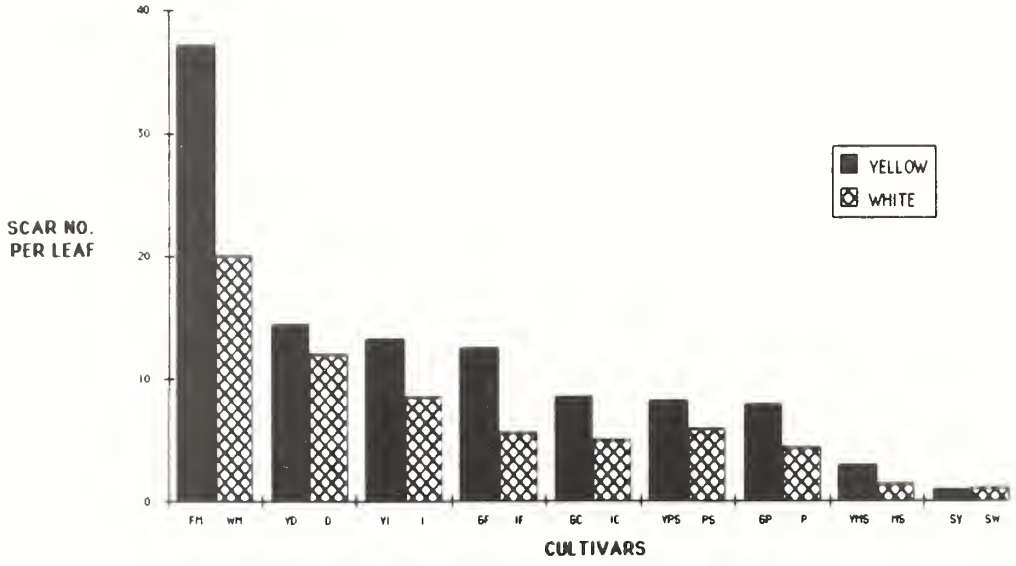

Figure 12. Feeding preferences of the WFT among yellow- and white-flowered sister chrysanthemum cultivars (See APPPENDIX for cultivar names).

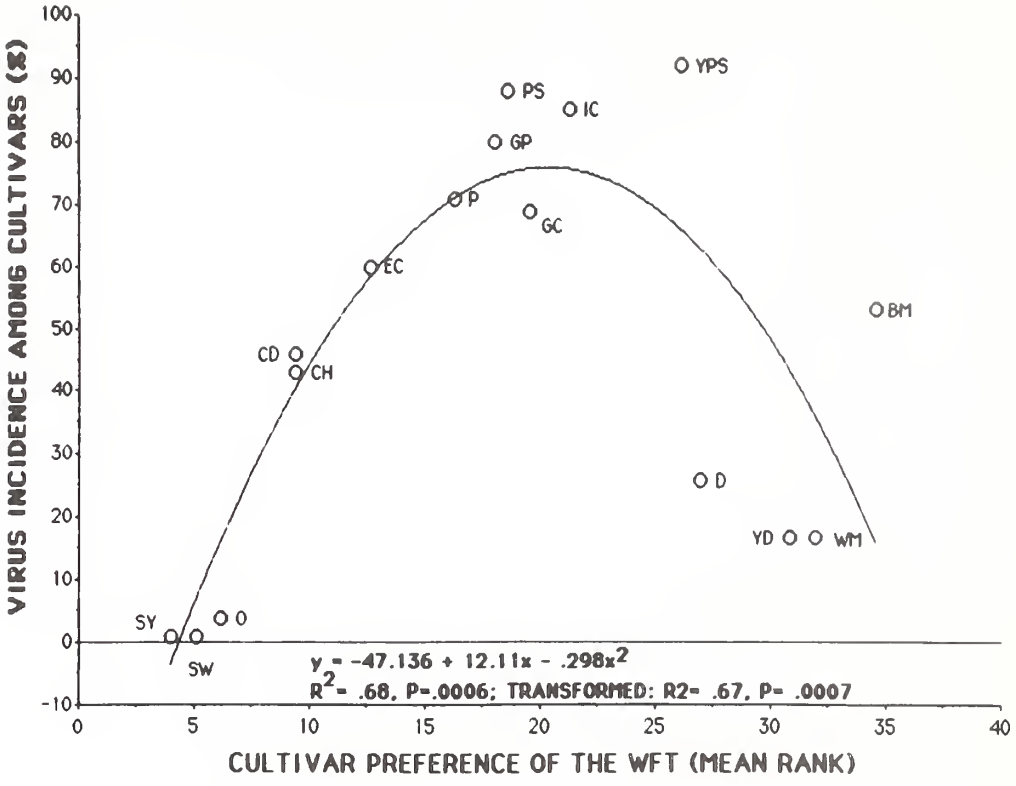

Figure 13. Correlation between cultivar preferences of the WFT and TSWV incidence among chrysanthemum cultivars in a commerical greenhouse.

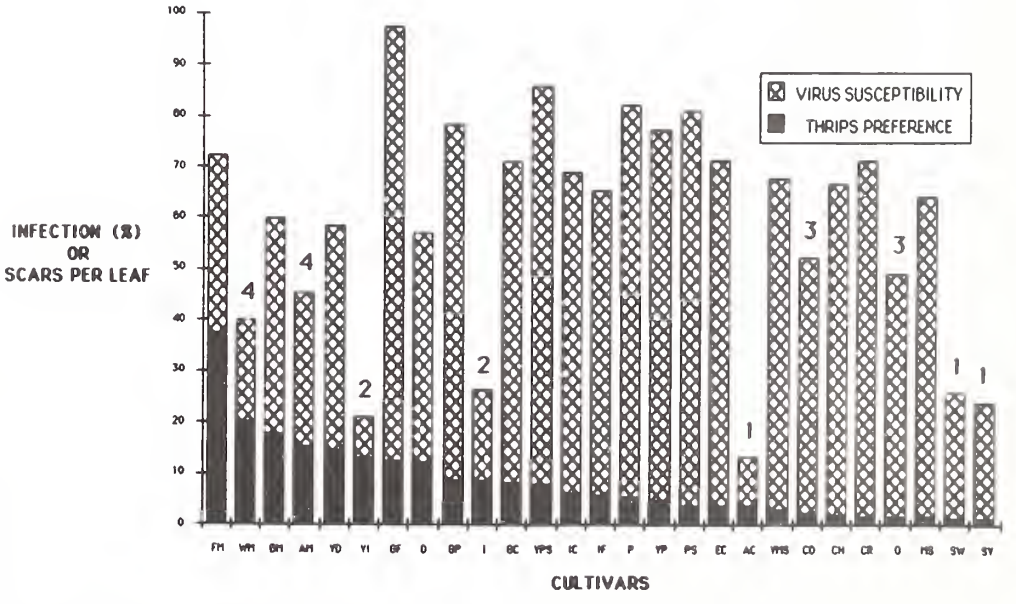

Figure 14. Comparison of virus susceptibility and thrips preference among chrysanthemum cultivars (See APPENDIX for cultivar names).

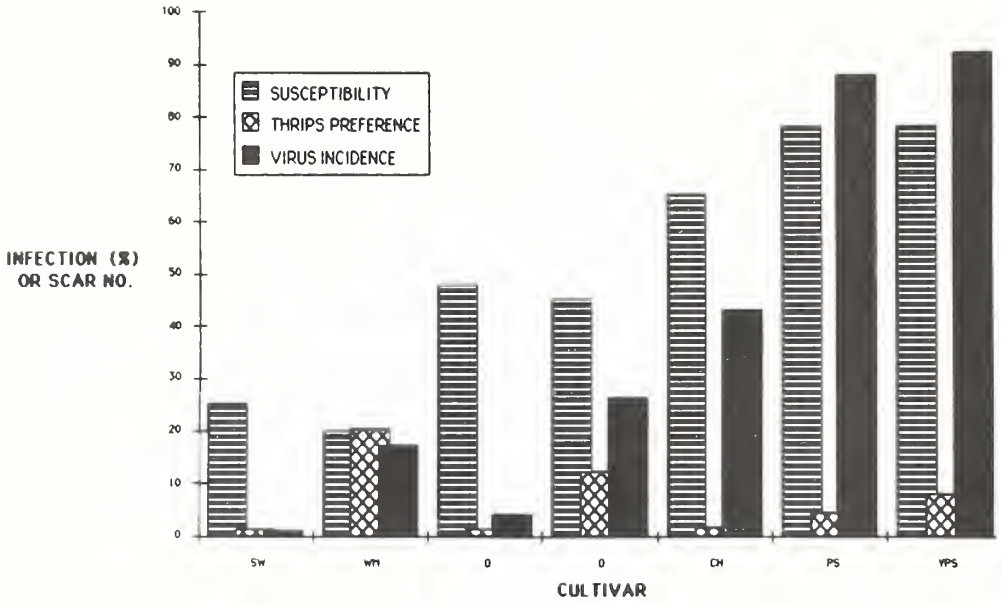

Figure 15. Influence of susceptibility to the TSWV and feeding preference on virus incidence in a commercial greenhouse (See APPENDIX for cultivar names).

EPIDEMIOLOGY OF TOMATO SPOTTED WILT VIRUS RELATIVE TO THRIPS POPULATIONS

FORREST L. MITCHELL AND J. W. SMITH, JR.

ABSTRACT

Thrips are common in peanut fields in Texas, and large populations appear on an annual basis. Until the advent of tomato spotted wilt virus (TSWV), thrips were not a pest in peanut and caused only cosmetic damage. However, thrips vectors are now responsible for millions of dollars of damage in south Texas fields. Major considerations in determining the relationship between the thrips and the TSWV epidemic include: determining thrips density and abundance, which thrips is an infective vector, which thrips species are present, and movement patterns of the species. In south Texas, it appears that the tobacco thrips, Frankliniella fusca, is the major vector. Although western flower thrips F. occidentalis are present, they do not appear to play as large a role in disease transmission in the peanut agroecosystem.

INTRODUCTION

Tomato spotted wilt virus (TSWV) has been known to occur in Texas since 1971, and perhaps as early as 1941 (Halliwell and Philley 1974). However, little notice was taken of the disease until 1985, when economic damage was incurred in the south Texas peanut fields of Frio and Atascosa counties. TSWV did not suddenly appear in 1985. Visiting plant pathologists noticed infection rates as high as 20% in south Texas peanut as early as 1983. In 1984, Texas Agricultural Experiment Station staff observed infection rates of 50% in some fields. Despite these observations, growers did not report yield losses, although losses may have occurred and been attributed to other causes. The economic losses perceived by growers in 1985 were due mostly to grade reduction of peanut harvested from infected fields rather than actual yield reduction. One symptom of TSWV in peanut is to redden the testa, an undesirable trait to buyers. Losses in 1986 were unquestionably due to crop destruction by the disease. Whole fields were ravaged, and few growers escaped the epidemic. Disease losses were not severe in 1987, and in 1988 and 1989 most fields had less than 1% TSWV infection.

Thrips are a common insect in peanut fields. Normally they feed and reproduce in terminals (folded quadrifoliates) and flowers, causing little but cosmetic damage. Research done in Texas and elsewhere (Smith and Sams 1977) has demonstrated no economic advantage to insecticidal control of thrips in peanut. The ecesis of tomato spotted wilt virus in Texas has changed this situation. The south Texas epidemic has been estimated to have caused $15 million in damages over a three county area.

Entomologists were faced with a paucity of available information on how to manage the epidemic, and consequently constructed a research program to collect data on: thrips species composition and relative abundance, which thrips species are vectors and the relative importance of each, and the role of alternate host plants. A summary of the research results and the current working hypothesis concerning the epidemic are presented here.

Mitchell is an Assistant Research Scientist with the Texas Agricultural Experiment Station, Route 2 Box 00, Stephenville, TX 76401, and Smith is a professor, Dept. of Entomology, Texas A&M University, College Station, TX 77843.

THRIPS SPECIES COMPOSITION AND RELATIVE ABUNDANCE:

Weekly sampling in south Texas peanut fields indicates that over five million thrips per acre may reside in a field over a season. Since each of these fields may exceed 150 acres, a large thrips population can exist in a field. Figure 1 shows an average field, with the total number of adults and immatures (all species) found in both flowers and terminals. In nine fields examined during 1987 and 1988 tobacco thrips, Frankliniella fusca, were the most common, representing over 80% of the adult population. Western flower thrips, F. occidentalis, were the next most abundant, representing about 18% of the population. The composite thrips, Microcephalothrips abdominalis, and the eastern flower thrips, F. tritici constituted <0.5% and <0.1% of the population respectively. Immatures are more common than adults, but are difficult to determine to species. Larval tobacco thrips and western flower thrips are virtually indistinguishable. Larvae collected from peanut and reared in the laboratory indicate that both tobacco thrips and western flower thrips reproduce in peanut fields, but tobacco thrips are more common. Other species of thrips were found during the study, but represent only single specimens and are likely to be accidental.

Both tobacco thrips and the western flower thrips are known vectors of TSWV (Sakimura 1962, 1963), while the eastern flower thrips is a proven non-vector (Sakimura 1953). The vectoring capability of the composite thrips is unknown. The onion thrips, Thrips tabaci, also a vector, was found in weed hosts on several occasions. However, it was rare and probably does not play a role in the peanut epidemic.

TRAP SAMPLING

Sampling of thrips populations with passive traps reveals interesting information about thrips movement. Figures 2 and 3 show the results of sticky trap captures from traps (cup traps similar to those used by Cho et al. 1987) placed on four foot stakes in survey fields. Western flower thrips are consistently captured in large numbers, and peak flight periods are discernible. Conversely, tobacco thrips are seldom captured and little can be deduced from the capture information, even though many millions were in the vicinity of the trap. Changing the color of the trap or the height of its placement (data not shown) did not affect the results. Water pan trap data yielded similar information. Wild host plant surveys (data not shown) have indicated that the western flower thrips is much more abundant than the tobacco thrips, a situation contrasting with the field data (Figure 1). The sticky trap data demonstrate that western flower thrips are a more mobile thrips, one perhaps better suited to the ephemeral wildflower habitat than the more sedentary tobacco thrips.

Thrips were also collected in soil emergence traps during January, February, and March of 1988. A trap consisted of a plastic pail with a clear petri dish inserted in the bottom. The pail was inverted over the ground and staked down. All traps were placed during on the soil during cold weather in January when thrips were inactive. The petri dish bottom (now the top) was smeared with a light coat of vaseline. Thrips emerged from the soil or litter beneath the pail, flew to the clear window and were captured in the vaseline. Table 1 shows the results of the capture. The western flower thrips to tobacco thrips ratio of 20 to 1 obtained with the aerial sticky traps now falls to less than 2 to 1. Stewart et al. (1989) sampled wild host plants in south Texas during the winter and were unable to locate tobacco thrips. Western flower thrips, however, were common throughout the winter. This leads to the assumption that the tobacco thrips is

Table 1.
Emergence trap captures - Pearsall, Texas 1988

Species	Number of traps		Number captured	
	In field	Out field	In field	Out field
Tobacco thrips	72	188	4	18
Western flower thrips	72	188	7	31
Composite thrips	72	188	12	30

in diapause and inactive in the soil and litter, and that warm weather during the winter will not cause it to become active. The western flower thrips becomes active during warm winter days and may or may not be in reproductive diapause.

ALTERNATE HOSTS FOR TSWV

The search for alternate wild hosts of TSWV in south Texas has proven fruitless. Very few plants have been found to have the disease, and these are isolated instances. Although random samples of plant species known to be able to harbor the disease were not taken, plants bearing possible symptoms were examined by enzyme linked immunosorbent assay (ELISA) and found to be negative. This is in contrast to areas such as Hawaii, where TSWV is found commonly in wild host plants, which serve as a reservoir for the disease (Cho et al. 1986). This observation is important in that it reduces the likelihood of TSWV epidemics in peanut being due to primary spread of the disease. Peanut is an annual crop, and TSWV must be annually introduced into the field. However, whether or not continued primary spread or the onset of secondary spread is most important to initiating the epidemic will determine the control tactics used to manage the disease.

DISEASE SPREAD

To test the hypothesis of disease spread, an insecticide exclusion experiment was conducted. Paired experimental blocks were designated in peanut fields during 1987. In each block, one plot was treated by growers under their normal management program, and one plot was left untreated by insecticide. The result was that in 24 experimental blocks (48 plots) TSWV prevalence was reduced from an average of 14% to an average of 8%, a 43% reduction. Figure 4 presents a summary from one of the fields. Even though insecticides were used heavily, thrips suppression was only moderate. Therefore, a portion of the remaining of virus infection in the control may represent secondary spread that was not reduced by treatment.

RELATIVE IMPORTANCE OF VECTOR SPECIES

During the growing season in 1987, thrips were collected from peanut plants bearing symptoms of TSWV. Uninfected plants were also collected and the thrips removed. Thrips were identified and examined individually by ELISA. Absorbance readings more than two standard deviations higher than the mean absorbance value for check thrips (thrips included on the ELISA plate that were known to be uncontaminated by TSWV), were considered positive for TSWV contamination. All peanut plants bearing TSWV symptoms were also analyzed by ELISA to ensure that the virus was indeed present. Although a contaminated thrips is not necessarily a vector, the summary in Table 2 is useful in that it provides a measure of

Table 2.
ELISA of thrips collected from peanut - Pearsall, Texas 1988

Species	Infected Plants	Uninfected Plants
Tobacco thrips	7/36 = 19.4%	40/381 = 10.5%
Western flower thrips	3/38 = 7.9%	33/433 = 7.6%
Immatures	24/159 = 15.1%	69/661 = 10.4%

exposure of thrips to TSWV. If the positive thrips collected from uninfected plants are considered as background and subtracted from the percent positive thrips removed from infected plants, then it is clear that tobacco thrips have more exposure to TSWV in the peanut agroecosystem. It is also clear that a thrips found on an infected plant has no guarantee of encountering TSWV.

CONCLUSIONS

Our current working hypothesis is that the tobacco thrips is the major vector of the disease in the south Texas peanut agroecosystem, and the western flower thrips plays a much lesser role. Not only are western flower thrips populations in peanut lower than those of the tobacco thrips, but it appears that those populations in the vicinity of the peanut agroecosystem have a lower exposure level to the virus. Additionally, the virus is rare in wild plant hosts, where the western flower thrips more commonly breeds. Thrips populations are always dense in peanut fields, so it is the population of infective vectors that matters. In this case, the number of infective western flower thrips appears low.

The peanut planting season in south Texas is long, with peanut plants being present from as early as March until as late as December. Since adult tobacco thrips appear to diapause and are inactive, there is no reason to assume that alternate host plants are necessary to maintain TSWV or thrips over the winter. Nor are alternate vectors necessary to introduce TSWV into the peanut fields each year in order to perpetuate the epidemic. Tobacco thrips emerging in the new year and carrying virus acquired in the previous year may remain alive long enough to inoculate the first peanut fields. If so, then the roots of the epidemic are based almost entirely in the peanut fields.

Research to test these hypotheses is continuing in our laboratories. As more data are gathered and analyzed in context with current information, we believe a clear picture of the epidemic will emerge.

ACKNOWLEDGMENT

The authors acknowledge the work of Mark Black, who has provided information on the presence of TSWV in wild host plants, and Brett Highland, who gathered much of the data in south Texas.

REFERENCES

Cho, J. J., R. F. L. Mau, D. Gonsalves, and W. C. Mitchell. 1986. Reservoir weed hosts of tomato spotted wilt virus. Plant Disease 70: 1014-1017.

Cho, J. J., W. C. Mitchell, R. F. L. Mau, and K. Sakimura. 1987. Epidemiology of tomato spotted wilt virus disease on crisphead

lettuce in Hawaii. Plant Disease 71: 505-508.

Halliwell, R. S. and G. Philley. 1974. Spotted wilt of peanut in Texas. Plant Dis. Reptr. 58: 23-25.

Sakimura, K. 1953. Frankliniella tritici, a non-vector of the spotted wilt virus. J. Econ. Entomol. 46: 915-916.

Sakimura, K. 1962. Frankliniella occidentalis (Thysanoptera: Thripidae), a vector of the tomato spotted wilt virus, with special reference to the color forms. Ann. Entomol. Soc. Am. 55: 387-389.

Sakimura, K. 1963. Frankliniella fusca, an additional vector for the tomato spotted wilt virus, with notes on Thrips tabaci, another vector. Phytopathology 53: 412-415.

Smith, J. W., Jr., and R. L. Sams. 1977. Economics of thrips control on peanuts in Texas. Southwestern Entomologist 2:149-154.

Stewart, J. W., C. Cole, and P. Lummus. Winter survey of thrips (Thysanoptera: Thripidae) from certain suspected and confirmed hosts of tomato spotted wilt virus in south Texas. J. Entomol. Sci. 24: 392-401.

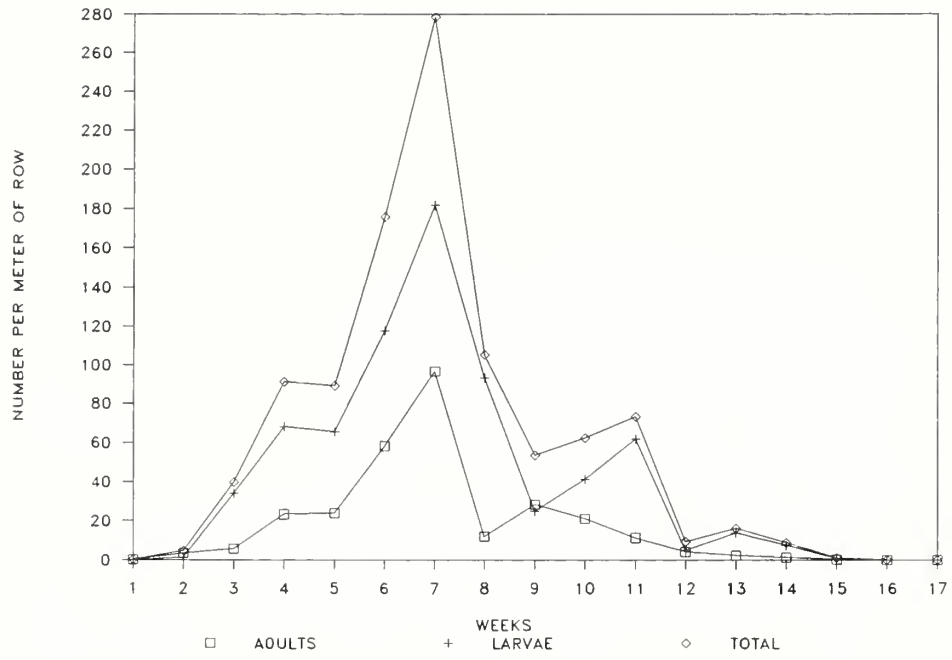

Figure 1
Weekly census of thrips in a peanut field.

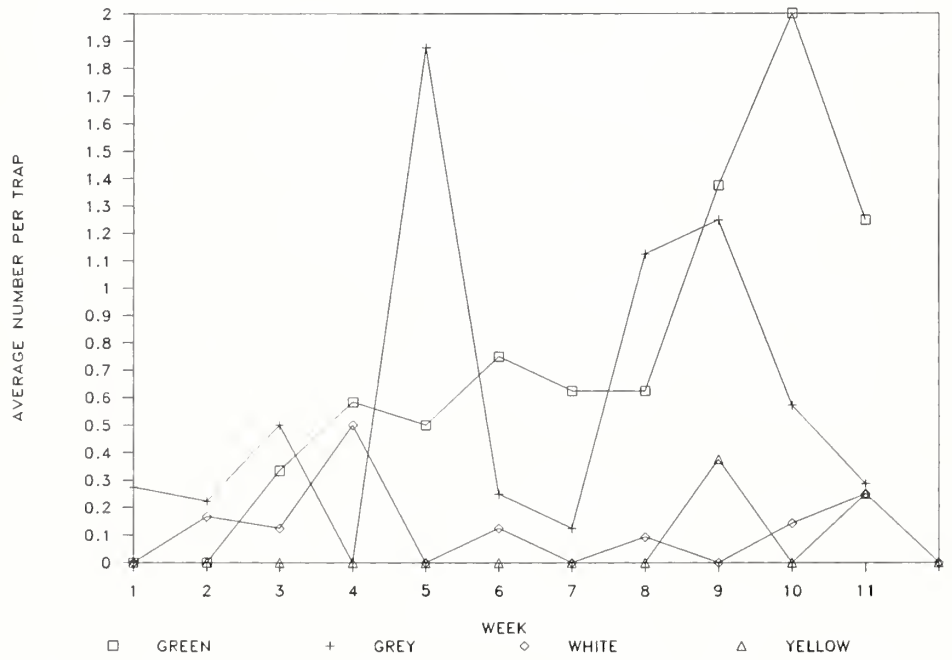

Figure 2.
Average number of tobacco thrips captured per trap per week.

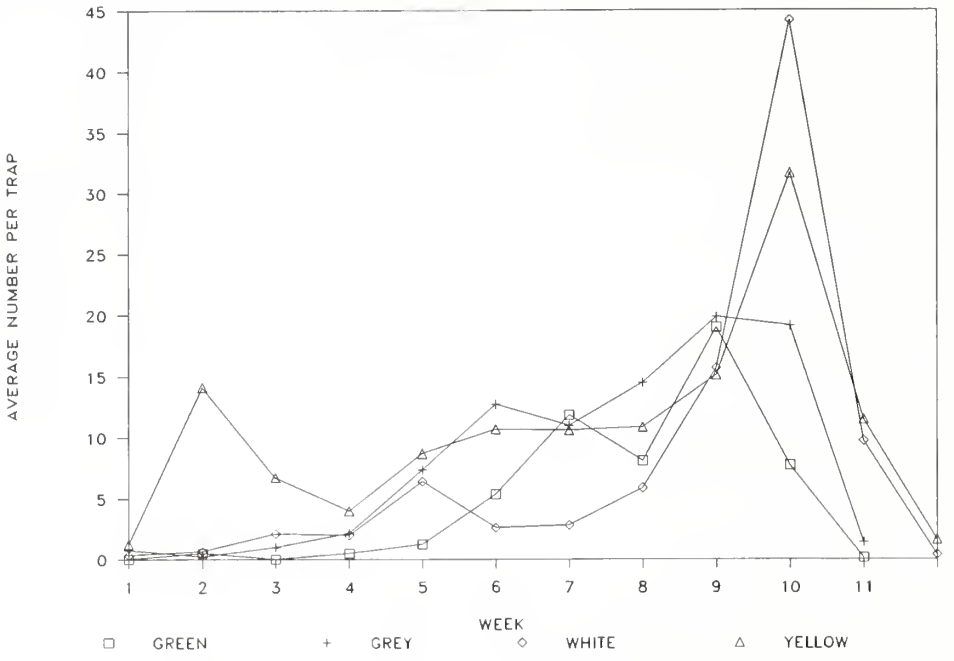

Figure 3.
Average number of western flower thrips captured per trap per week.

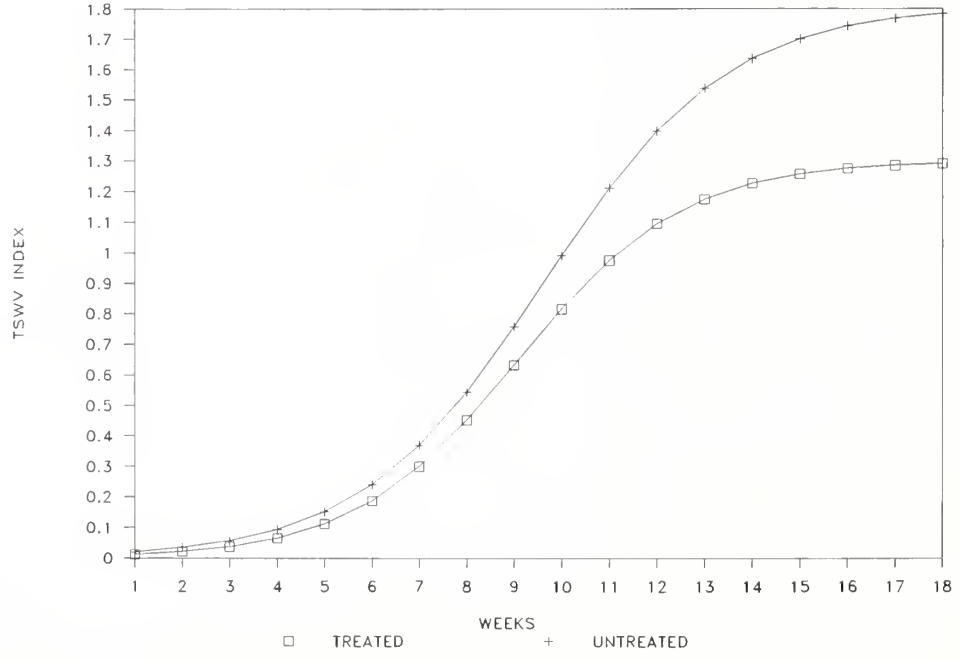

Figure 4.
Prevalence curves for tomato spotted wilt virus in treated and untreated field plots.

CHARACTERIZATION OF A SEROLOGICALLY DISTINCT TSWV-LIKE VIRUS FROM IMPATIENS

J. W. Moyer, M. D. Law, and L. A. Urban

ABSTRACT

A TSWV-like virus (TSWV-I), which reacted poorly with TSWV antiserum, was isolated from the hybrid flower crop, New Guinea impatiens and later was found to be widespread in floral crop production in the United States. Three RNA's were isolated from purified virions of the TSWV-like isolate which comigrated with RNA's of known TSWV isolates. Hybridization analysis under moderately high stringency conditions revealed no hybridization between clones of TSWV-I S and M and the S and M RNA's of TSWV, respectively. Serological analysis demonstrated that while there was conservation of epitopes on the envelope proteins, G1 and G2, no serological relatedness was detected between the nucleocapsid proteins of TSWV and TSWV-I. Enveloped virions were relatively rare in TSWV-I infected tissue when assayed by immunosorbent electron microscopy or when tissues were examined in ultrathin section by electron microscopy. Paracrystalline arrays of filamentous structures were abundant in the TSWV-I infected tissue. These properties suggest that TSWV-I should be the type member of a second serogroup in the tomato spotted wilt virus group.

INTRODUCTION

The tomato spotted wilt virus group of plant viruses is an enigma among the groups of viruses which infect plants. Tomato spotted wilt virus (TSWV) is known to occur worldwide with a host range that spans across many families of dicotyledons as well as a few monocotyledons. It has long been considered a monotypic group in that only one virus has been identified (Ie, 1970; Francki et al., 1985) although many stable variants have been described which express phenotypic differences in biological characters such as symptomatology (Best, 1968). These variants have not been further characterized at the molecular or even serological level. Indeed, only recently has progress been made in the molecular characterization of a single isolate of this virus (DeHaan et al., 1989). Although a molecular characterization has been conspicuously absent, TSWV is generally considered to most closely resemble the Bunyaviridae family of animal viruses due to the similarity in physicochemical properties (Milne and Francki, 1984). More recent evidence supports this hypothesis and further suggests that TSWV most closely resembles the phlebovirus genus of that family (DeHaan et al., 1988; DeHaan et al., 1989). Virions are quasi-spherical particles ranging from 80 to 120 nm in diameter. The virion consists of an outer envelope which contains two glycoproteins of 78K and 58K Mr which have been designated G1 and G2, respectively. A third protein of about 28K Mr, designated N, is found in the interior of the virion along with small amounts of an L protein (approximately 120K Mr) (Mohamed et al., 1973; Tas et al., 1977).

Our interest in TSWV has been to characterize the diversity among isolates of TSWV which infect floral crops. This research was initiated to determine the basis of the difficulty encountered in serologically diagnosing TSWV in floral crops. We and others have tested plant samples which expressed symptoms typical of TSWV in their natural host as well as in experimental hosts. Serological reactions using antisera produced with known isolates of TSWV reacted strongly against some isolates and gave weak or no reactions against other samples obtained from plants expressing similar symptoms. These samples also tested negative for tobacco mosaic virus, tobacco streak virus and turnip mosaic virus which are reported to infect these crops, especially impatiens. The isolates which reacted weakly with the TSWV

Moyer, Law and Urban are Professor, Graduate Assistant, Post-doctoral Research Associate, respectively, Department of Plant Pathology, North Carolina State University, Raleigh, North Carolina 27695-7616.

antisera were predominantly from floral crops such as various types of impatiens, begonia, dahlia, exacum, gloxinia as well as several others.

RECOGNITION OF SEROLOGICALLY DISTINCT TSWV-LIKE ISOLATES

In preliminary investigations, isolates which did and did not react with common TSWV antiserum were compared on a host range of gloxinia, impatiens and Nicotiana benthamiana. Only minor differences in symptom expression were observed between isolates on a given host. Further, the range of differences observed among those isolates which did react with the TSWV antisera was similar to the type and range of symptoms observed for the isolates which did not react. Strong and weak (or null) serological reactions were generally similar across hosts. Consistent serological reactions by ELISA or dot-immunobinding assay were obtained by testing the isolates from N. benthamiana. When these same samples were analyzed for serological reactivity to the individual structural proteins by Western analysis we observed that the glycoproteins from all of the isolates reacted weakly to the antiserum from the common strain of TSWV, including those isolates which had reacted weakly by the other tests. The N protein was only detected from the isolates which had reacted strongly to the TSWV antiserum in the other tests. The N protein was not detected in extracts for any of the weak reacting isolates. Differences in the degree of conservation of serological reactions between the structural proteins is the basis by which serogroups within genera are defined in the Bunyaviridae (Shope, 1985; Elliott, 1990). Only a single preliminary report of serological divergence among TSWV isolates has been reported for an isolate originating in watermelon. This isolate was also shown to have a serologically distinct N protein (Kameyi-Iwaki et al., 1988); however, no additional information was provided.

Further observations have revealed that this variant of TSWV is the predominant TSWV-like pathogen of floral crops in the United States (Personal communication R. K. Jones, North Carolina State University; R. Davis, Agdia). We have also shown that this population of variants should be classified as the type member of a second serogroup in the tomato spotted wilt virus group of plant viruses (Law and Moyer, 1990). Here we have reviewed the other biochemical and cytopathic characteristics of this virus we have provisionally designated TSWV-I until a permanent name can be given.

BIOCHEMICAL PROPERTIES AND RELATIONSHIP TO TSWV

Purified preparations of TSWV-I (Law and Moyer, 1990) contained proteins which comigrated with N and G2 proteins from purified preparations of common TSWV (Fig 2a). Although G1 was not observed in purified preparations, it was detected in tissue extracts from TSWV-I infected plants analyzed by Western transfer and probed with TSWV G1 antiserum (Fig 2e). Analysis of the nucleic acid under denaturing conditions revealed three RNA's from TSWV-I which comigrated with purified RNA's from the common strain (Fig 3). These results indicated that the serologically distinct isolate (TSWV-I) was structurally similar to the common serotype of TSWV.

Antibodies to purified TSWV-I reacted against proteins which comigrated with N and G2, but not G1 since it was absent from the purified preparations used as the immunogen (Fig 2b). G2 was only detected in gels overloaded with virus. This antiserum did not detect the common isolates of TSWV in western blots, ELISA, or by immunosorbant electron microscopy. The TSWV-I antiserum did trap a relatively small number of enveloped virions and filaments typical of particles of ribonucleoprotein. Additional serological comparisons of the two serotypes were made using antisera raised to each of the individual proteins (G1, G2 and N) purified from a common strain of TSWV. Antisera to G1 and to G2 detected G1 and G2, respectively, in tissue infected with either TSWV or TSWV-I (Fig 2d & e). These antisera were also used to trap enveloped particles for immunosorbant electron microscopy. Although the antisera trapped virions from tissue infected with either isolate, it was 8 to 10 fold less efficient in trapping enveloped particles in the tissue infected with TSWV-I. Antiserum to N protein only detected the N protein in TSWV-infected tissue and not in the TSWV-I infected tissue similar to the antiserum made against virions of TSWV. These experiments provide evidence for the presence of conserved epitopes on the G1 and G2

proteins of TSWV and TSWV-I. There was no relationship detected between the N proteins of these two viruses and provides evidence for serological distinctions among TSWV-like viruses (Law and Moyer, 1990; Wang and Gonsalves, 1989). The conservation of epitopes between the glycoproteins is an additional character used to define serogroups within the phlebovirus genus of the bunyaviridae (Shope, 1985; Elliott, 1990).

RNA isolated from purified virions of TSWV (Gonsalves and Trujillo, 1986) and from TSWV-I (Law and Moyer, 1990) was used as template for synthesis of cDNA's. The purified RNA's of TSWV-I comigrated with purified RNA from TSWV. Thus, demonstrating that TSWV-I is not a deletion variant defective for assembly as was previously reported for isolates of TSWV which did not produce mature virions (Ie, 1982; Verkleij and Peters, 1983). Two libraries were produced in the commercially available vector Lambda Zap (Stratagene) and clones were isolated from each library for the respective S and M RNA's. When these clones were used to probe Northern blots of the RNA's it was revealed that under conditions of high stringency (65 C, 0.1x SSC) the cDNA's from one isolate did not cross hybridize in reciprocal hybridizations of the RNA's. These results are consistent with attempts to detect isolates representative of the two serotypes in infected N. benthamiana. Preliminary sequence analysis of TSWV and TSWV-I has confirmed the previous report by DeHaan et al. (1989) of an 8 nucleotide terminal consensus sequence, 5' d(GAUUGCUC), on both the S and M RNA's of TSWV. This sequence has been followed (3' terminal) by a dU nucleotide in the clones with terminal sequence which we have analysed. We have also observed this sequence on the M RNA of TSWV-I. At this writing the consensus sequence detected on the S RNA of TSWV-I is variable and no conclusions have been reached. Although sequencing of these RNA's is incomplete, significant heterogeneity exists between the sequences immediately 5' to the consensus sequence of the corresponding RNA's of TSWV and TSWV-I. This was expected from the hybridization analysis of the northern blots.

COMPARATIVE CYTOPATHOLOGY OF TSWV AND TSWV-I

The quasi-spherical, enveloped virions typical of TSWV were only rarely observed in electron micrographs of N. benthamiana tissue infected with TSWV-I whereas, the virions were prevalent in tissues infected with a typical isolate of TSWV (Fig 4). The relative lack of mature virions observed in TSWV-I infected tissue was consistent with the small number of virions detected by immunosorbent electron microscopy in the TSWV-I relative to the TSWV-infected tissues. Although there was a scarcity of virions in the TSWV-I tissue, there was a significant abundance of electron dense granular material and numerous filaments (Fig 4). The filaments were arranged in paracrystalline arrays which in cross-section appeared as alternating rows of spheres (10 - 20 nm dia) and filaments. When viewed at an oblique angle these arrays resembled stacks of Z-shaped structures (Law and Moyer, 1990) This observation revealed that the spherical structures were the ends of the filaments. The paracrystalline structures were sensitive to some embedding media, such as LR-White, indicating a somewhat delicate structure. The granular, amorphous, electron dense material and filamentous structures have been previously reported in tissues infected with the typical strain of TSWV (Francki and Grivell, 1970; Kitajima, 1965; Milne, 1970).

A comparative study of N. benthamiana infected with either TSWV or TSWV-I revealed that the virus-associated structures for both types were membrane associated and occurred in the cytoplasm. The membrane association was not always evident in cells containing the paracrystalline structures which accumulated to high concentrations. Structures for both TSWV and TSWV-I were observed in vascular parenchyma, mesophyll, palisade and epidermal tissues.

No cell types were found to be uniquely infected by either strain. Onset of symptoms, detection of viral proteins and appearance of viral related structure were correlated in both strains. Disease progression was rapid in both types. Cytological evidence suggests that TSWV-I may be largely in the form of inclusions.

CONCLUSIONS

We believe that TSWV-I should be designated as a virus distinct from TSWV and that it should be the type member of a new serogroup designated I. This classification is consistent with the criteria used for delineating serogroups within the genus Phlebovirus of the animal virus family Bunyaviridae.

REFERENCES

Best, R. J. 1968. Tomato spotted wilt virus. Advances in Virus Research 13:65-146.

De Haan, P., Wagemakers, L., Goldbach, R., and Peters, D. 1988. Tomato spotted wilt virus: A Bunyavirus in plants. Virus Research Supplement 2:83.

De Haan, P., Wagemakers, L., Peters, D., and Goldbach, R. 1989. Molecular cloning and terminal sequence determination of the S and M RNA's of tomato spotted wilt virus. J. of General Virology 70:3469-3473.

Elliott, R. M. 1990. Molecular biology of the Bunyaviridae. J. of General Virology 71:501-522.

Francki, R. I. B., and Grivell, C. J. 1970. An electron microscope study of the distribution of tomato spotted wilt virus in systemically infected Datura stramonium leaves. Virology 42:969-978.

Francki, R. I. B., Milne, R. G., and Hatta, T. 1985. Tomato spotted wilt virus. In Atlas of Plant Viruses, Volume 1, pp. 101-110. Boca Raton, Fl.: CRC Press.

Gonsalves, D., and Trujillo, E. E. 1986. Tomato spotted wilt virus in papaya and detection of virus by ELISA. Plant Disease 70:501-506.

Ie, T. S. 1970. Tomato spotted wilt virus. CMI/AAB Descriptions of Plant Viruses No. 39. Commonwealth Mycological Institute, Kew.

Ie, T. S. 1982. A sap-transmissible, defective form of tomato spotted wilt virus. Journal of General Virology 59:387-391.

Kameyi-Iwaki, M., Hanada, K., Honda, Y., and Tochihara, H. 1988. A watermelon strain of tomato spotted wilt virus (TSWV-W) and some properties of its nucleocapsid (Abstract). 5th International Congress of Plant Pathology. Kyoto, Japan.

Katajima, E. W. 1965. Electron microscopy of viracabeca virus (Brazilian tomato spotted wilt virus) within the host cell. Virology 26:89-99.

Law, M. D., and Moyer, J. W. 1990. A tomato spotted wilt-like virus with a serologically distinct N protein. J. General Virology 71:933-938.

Milne, R. G. 1970. An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. Journal of General Virology 6:267-276.

Milne, R. G., and Francki, R. I. B. 1984. Should tomato spotted wilt virus be considered as a possible member of the family Bunyaviridae? Intervirology 22:72-76.

Mohamed, N. A., Randles, J. W., and Francki, R. I. B. 1973. Protein composition of tomato spotted wilt virus. Virology 56:12-21.

Shope, R. E. 1985. Bunyaviruses. In Virology, pp. 1055-1082. Edited by B. N. Fields et al. New York: Raven Press.

Tas, P. W. L., Boerjan, M. L.,and Peters, D. 1977. The structural proteins of tomato spotted wilt virus. Journal of General Virology 36:267-279.

Verkleij, F. N., and Peters, D. 1983. Characterization of a defective form of tomato spotted virus. Journal of General Virology 64:677-686.

Wang, M., and Gonsalves, D. 1990. ELISA detection of various tomato spotted wilt virus isolates using specific antisera to structural proteins of the virus. Plant Dis. 74:154-158.

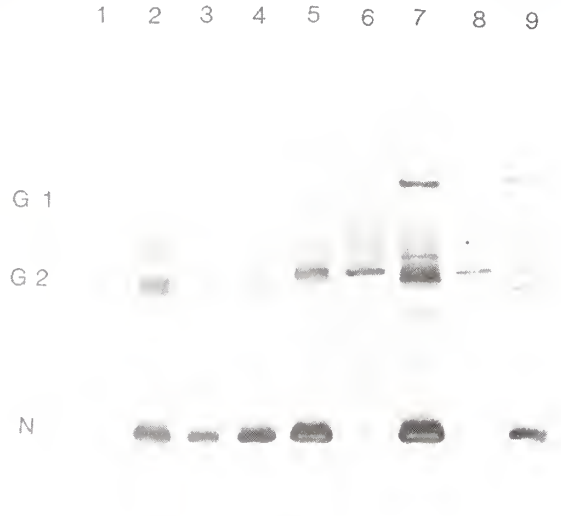

Figure 1. Western blot analysis of TSWV and TSWV-like isolates from various plant sources in extracts of Nicotiana benthamiana using TSWV polyclonal antibodies. Lane 1, healthy plant; lanes 2, 3, 4, 5, 7, and 9 contain extracts from isolates originating in tobacco, dahlia, tomato, lettuce, impatiens and begonia, respectively, that give typical reactions to TSWV antiserum; lanes 6 and 8 contain extracts from isolates originating in impatiens and gloxinia that have the N protein which is not recognized by the TSWV-I antiserum.

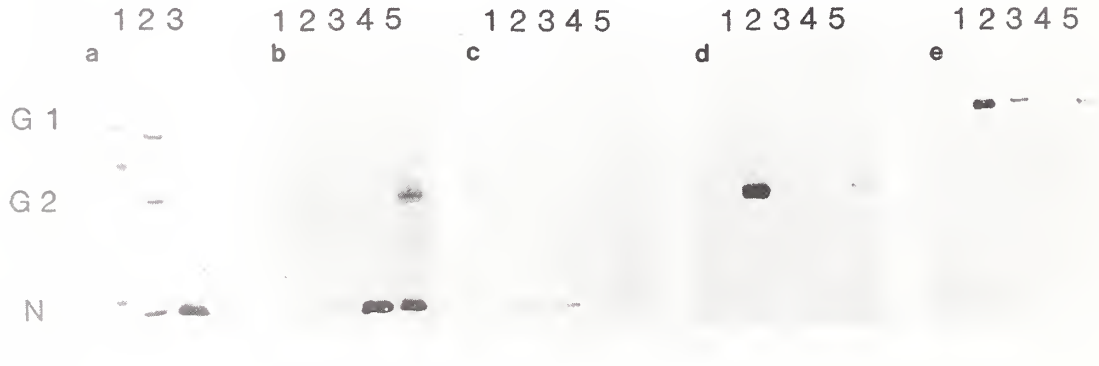

Figure 2. Serological analysis of purified TSWV and TSWV-I proteins by Western blotting. Panel (a) is a Coomassie blue-stained SDS-PAGE gel; lane 1, molecular weight markers; lane 2, dissociated TSWV virions; lane 3, dissociated purified TSWV-I preparations. Panels (b), (c), (d), and (e) were blots probed with antibodies to TSWV-I, TSWV N protein, TSWV G2 protein and TSWV G1 protein. Lane 1, healthy N. benthamiana tissue extract; lane 2, purified TSWV virions; lane 3, TSWV-infected N. benthamiana tissue extract; lane 4, purified TSWV-I virions; lane 5, TSWV-I infected N. benthamiana tissue extract (Law and Moyer, 1990).

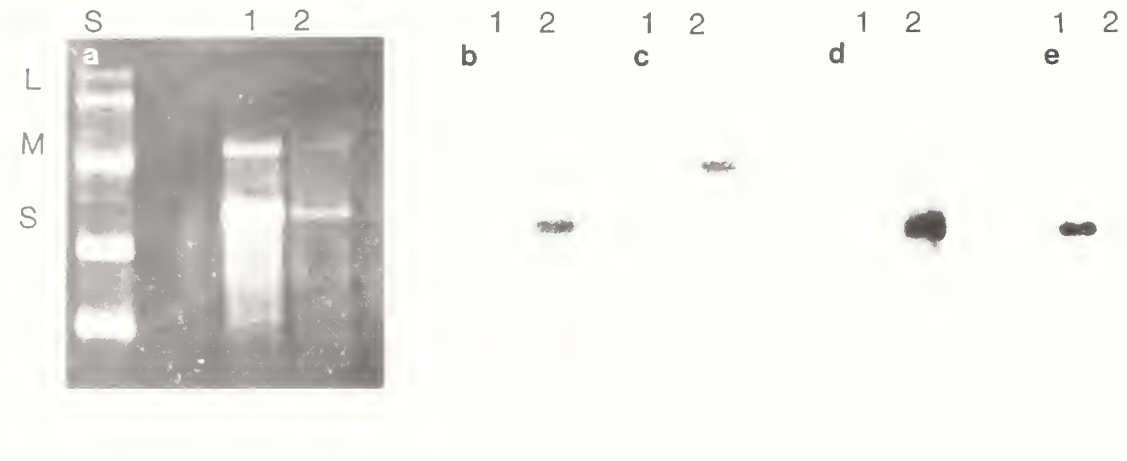

Figure 3. Size and hybridization analysis of TSWV and TSWV-I RNAs electrophoresed in denaturing conditions. Panel (a) Ethidium bromide-stained glyoxal agarose gel. Lane S contains Mr standards; 0.24 to 9.5 kb RNA ladder (Bethesda Research laboratories). Lanes 1 and 2 contain purified TSWV and TSWV-I RNA, respectively. Panels (b), (c), (d), (e) Northern blots hybridized with ^{32}P-labelled nick-translated TSWV-I S-specific clone, TSWV-I M specific clone, TSWV-I S and M specific clones, and TSWV M specific clone, respectively (Law and Moyer, 1990).

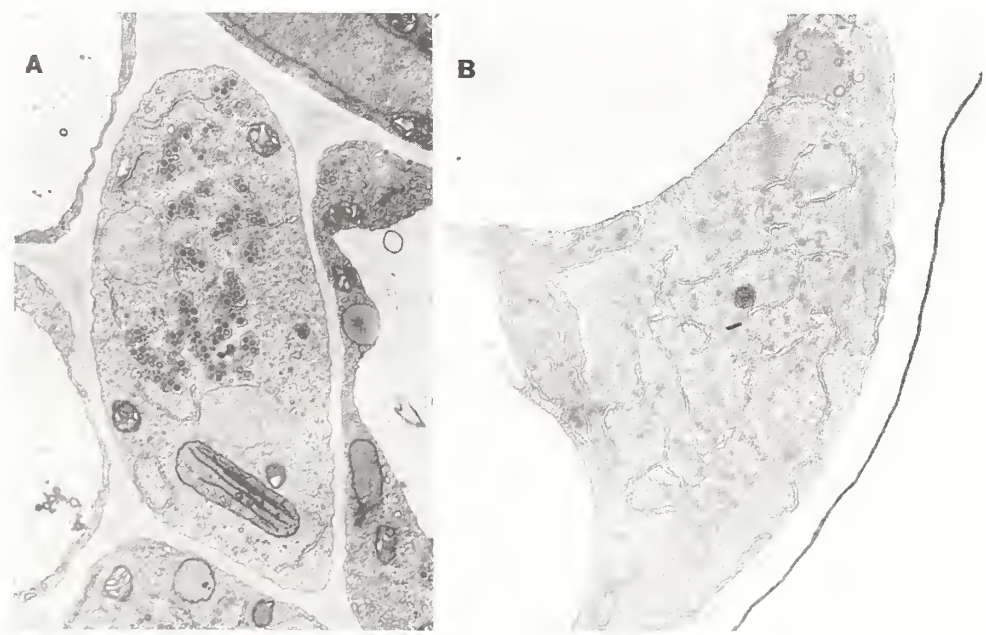

Figure 4. Electron micrographs of TSWV and TSWV-I infected Nicotiana benthamiana. Panel A is an example of a vascular parenchyma cell infected with TSWV. Note enveloped virions in membrane bound bundles. Panel B is a TSWV-I infected cell containing electron dense granular material, fibrillar paracrystalline structures and virions.

GENETIC ORGANIZATION AND EXPRESSION OF THE TOMATO SPOTTED WILT VIRUS GENOME

Peter de Haan, Richard Kormelink, Dick Peters and Rob Goldbach

Department of Virology, Agricultural University,
Binnenhaven 11, P.O.B. 8045,
6700 EM Wageningen, The Netherlands

ABSTRACT

The nucleotide sequence and genetic organization of the S and L RNA segments of tomato spotted wilt virus (TSWV) indicate that this virus represents a bunyavirus unique in its property to infect plants. Partial sequence data, obtained from a naturally occurring, shortened version of the L RNA (denoted L' RNA), show that L RNA is negative stranded, the viral complementary (vc) strand containing one large open reading frame. The N-terminal sequence of the primary translation product of this genome segment shares some sequence motifs with the L protein encoded by Bunyamwera virus, the prototype of the family Bunyaviridae. The S RNA is 2916 nucleotides long and has an ambisense character, containing one ORF in the viral strand and another in the vc strand. This genome arrangement is similar to that of S RNA of phlebo- and uukuviruses, two genera of the Bunyaviridae. In vitro transcription and translation experiments identified the ORF in the vc strand of S RNA to correspond with the nucleocapsid protein gene. Expression of the viral sense, second ORF in S RNA, using the baculovirus expression system, resulted in high level synthesis of a non-structural protein (NSs) to which antibodies were raised. Using this antiserum in Western blot and immunogold analysis the corresponding viral protein was detected in infected *N. rustica* tissues.

INTRODUCTION

Among plant viruses tomato spotted wilt virus (TSWV) fills a unique taxonomic position. The virus has a distinct particle morphology and genome structure and is sofar the only plant virus unequivocally demonstrated to be transmitted by thrips (*Thysanoptera*). Hence it was plausible to classify TSWV as the sole repre-

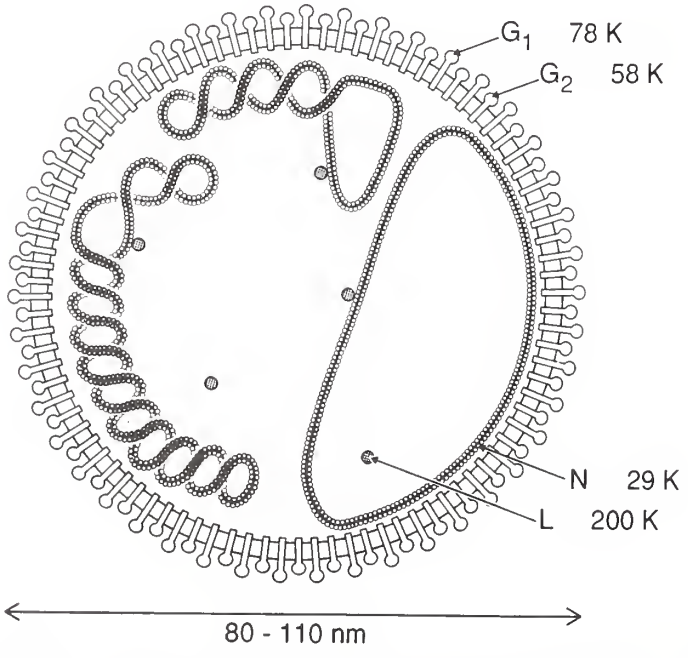

Figure 1. Schematic representation of a TSWV particle. The three linear RNA segments are tightly associated with nucleocapsid (N) proteins and form circles, which may be coiled. The lipid envelope contains two species of glycoproteins, G1 and G2. A large protein (L), present in minor amounts, may represent the viral transcriptase.

sentative of a distinct group, the tomato spotted wilt virus group (Ie, 1970; Matthews, 1982). The virion of TSWV is a spherical membrane-bound particle, 80-110 nm in diameter, covered with surface projections (Fig. 1). Four different proteins can be distinguished: an internal nucleocapsid protein (N) of 29 kilodaltons (K), two envelope glyco-proteins (G1 and G2) of 78K and 58K respectively, and a large protein (L) of approximately 200K (Mohamed *et al*, 1973; Tas *et al*, 1977). The genome consists of three linear single-stranded RNA molecules of approximately 3000 nucleotides (S RNA), 5000 nucleotides (M RNA) and 8300 nucleotides (L RNA). These genome segments are tightly complexed with N proteins and form circular nucleocapsids (Fig. 1 and Van den Hurk *et al*, 1977; De Haan *et al*, 1989a).

Based on the properties summarized here it has been proposed that TSWV actually represents a member of the large family of arthropod-borne Bunyaviridae, being unique in its property to infect plants (Milne and Francki, 1984; De Haan *et al*, 1989a). To gain more insight in the molecular genetic properties of TSWV and to

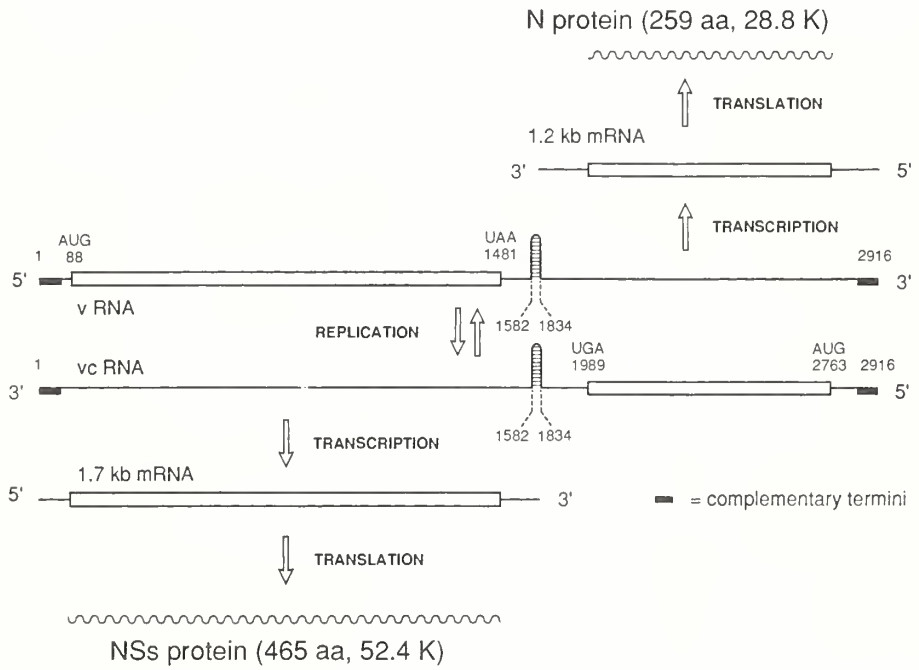

Figure 2. Genetic organization and expression of TSWV S RNA. The RNA is 2916 nucleotides long and has an ambisense coding strategy. The ORF in the viral strand (vRNA) encodes a 52.4K non-structural protein (NSs), the ORF in the viral complementary strand (vc RNA) represents the nucleocapsid protein (N) gene. The ORFs are expressed from subgenomic mRNAs of 1.7 and 1.2 Kb. Numbers refer to nucleotide positions, the black bars represent the complementary termini. For further details see text.

classify this virus properly, detailed information on its genome is required. Therefore we decided to clone and sequence the genome of TSWV (De Haan *et al*, 1989b, 1990a). In this paper the molecular organization and expression of the L and S RNA segments are discussed.

STRUCTURE AND EXPRESSION OF TSWV S RNA

Using a series of cDNA clones covering more than 95% of the S RNA (De Haan *et al*, 1989b) the major part of this genome segment was sequenced. Terminal sequences not included in these clones were elucidated by primer extension (De Haan *et al*, 1990a). The complete sequence of S RNA is 2916 nucleotides long and has the following characteristics (Fig. 2):

(i) The 3'- and 5'-terminal sequences are complementary over a stretch of 65 to 70 nucleotides and can be folded in a stable panhandle structure with a free energy of G = -254.1 kJ/mol. The formation of such panhandle may explain the pseudo-circular state of the nucleocapsids (Fig. 1).

(ii) The RNA has an ambisense coding strategy, containing one open reading frame (ORF) in the viral strand, and a second ORF in the complementary strand (Fig. 2).

(iii) A 125 basepair-long hairpin is present in the intercistronic region.

The ORF on the viral strand starts with an AUG codon at position 88 and terminates at an UAA termination codon at position 1481, corresponding with a protein of 465 amino acids and a size of 52.4K (Fig. 2). The ORF on the viral complementary strand starts with an AUG codon at position 2763 (numbered from the 5' end of the viral strand) and terminates at an UGA stop codon at position 1989, corresponding with a protein of 259 amino acids and a size of 28.8K. Although both proteins do not exhibit any sequence homology to proteins encoded by any bunyavirus (and neither to any other protein in the EMBL protein and nucleotide sequence database) the genetic organization of TSWV S RNA, with two genes in ambisense arrangement and complementary termini, strikingly resembles that of the S RNA of phleboviruses (Ihara *et al*, 1984) and uukuviruses (Simons *et al*, 1990) (both family Bunyaviridae) and Pichinde virus (family Arenaviridae) (Romanowski *et al*, 1985). In addition, as also found for Punta Toro phlebovirus (Emery and Bishop, 1987) an inverted complementary A-U rich sequence is located in the intercistronic region of TSWV S RNA, which can be folded into a stable hairpin structure of approximately 125 basepairs (Fig. 2 and De Haan *et al*,

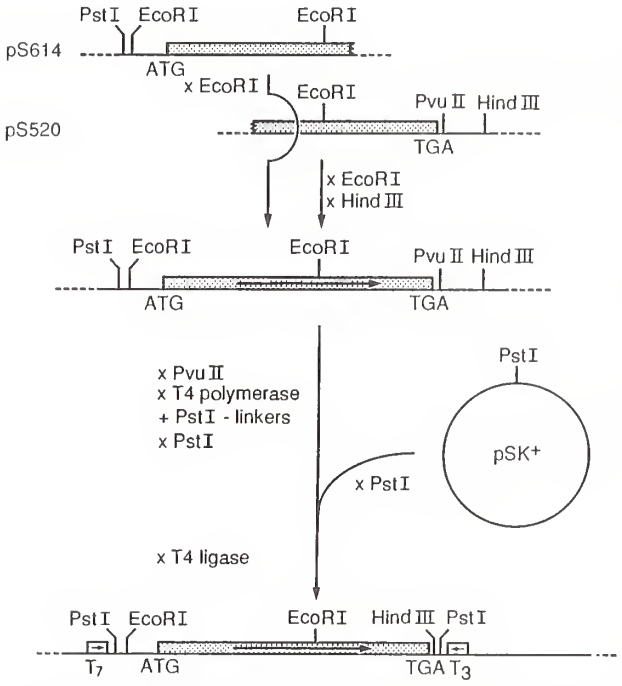

Figure 3. Cloning of the nucleocapsid protein gene in transcription vector pSK+ (Stratagene). Using S RNA-specific cDNA clones pS614 and pS520 the ORF residing in the vc strand (see Fig. 2) was reconstructed and cloned in vector pSK+ downstream of the phage T7 promoter. The resulting construct pTSWV-vcORF was used for <u>in vitro</u> expression studies (see Fig. 4).

1990a). This structure may act as a transcription termination signal as it was found (De Haan *et al*, 1990) that the two ORFs are expressed from two subgenomic mRNAs (Fig. 2). Using single stranded m13 and riboprobes a subgenomic mRNA of 1.7 Kb was detected, corresponding to the large ORF, and a subgenomic mRNA of

1.2 Kb corresponding to the short ORF, in both infected *Nicotiana rustica* as well as in tomato. To analyze and to identify the two proteins encoded by S RNA the two ORFs were cloned and translated either *in vitro* or *in vivo*. The length of the ORF in the viral complementary strand corresponds well with the size of the nucleocapsid protein (29K apparent size as deduced from electrophoretic mobility). Indeed this ORF could be identified as the nucleocapsid protein gene. To this end the ORF was reconstructed using cDNA fragments of clones pS614 and pS520 and cloned in transcription vector pSK$^+$ (Fig. 3). The resulting construct (pTSWV-vcORF) was linearized with Hind III and run-off transcripts were produced using T7 RNA polymerase. Upon translation of these transcripts in a rabbit reticulocyte lysate a discrete product was obtained (Fig. 4. lane 3), which comigrated with isolated nucleocapsid protein and, moreover, was recognized by antibodies raised against purified nucleocapsids (Fig. 4, lane 5). Hence the 28.8K ORF in S RNA corresponds to the nucleocapsid protein gene.

The second ORF in S RNA probably corresponds to a nonstructural protein, which we propose to refer to as NSs, according to the bunyavirus nomenclature. The coding function of this ORF was studied by expression in insect cells using a baculovirus vector. Using transfer vector pAC33DZ1 (Zuidema *et al*, 1990) the ORF was cloned downstream of the polyhedrin promoter of baculovirus AcMNPV. In-

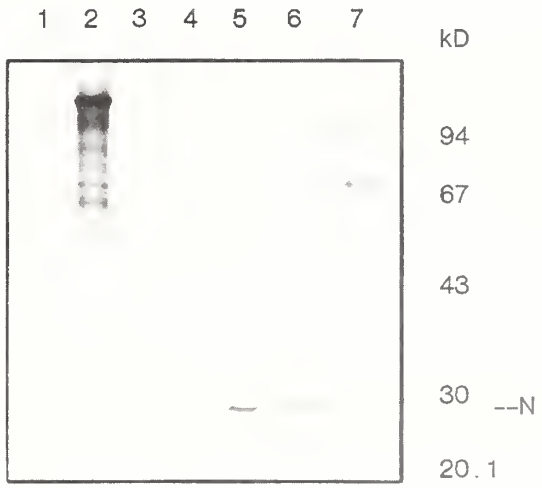

Figure 4. Identification of the nucleocapsid protein gene by *in vitro* translation of transcripts from plasmid pTSWV-vcORF. RNAs were translated using the rabbit reticulocyte lysate. Samples were: lane 1, control (no RNA added); lanes 2 and 4, translation product of TMV RNA (major product 126K); lanes 3 and 5, translation products directed by pTSWV-vcORF. Translation products were analyzed directly (lanes 1 to 3) or immunoprecipitated using polyclonal antibodies against purified nucleocapsids prior to electrophoresis (lanes 4 and 5). Lanes 6 and 7 contain Coomassie Blue-stained purified nucleocapsids and marker proteins, respectively. Mr. values are indicated on the right.

fection of *Spodoptera frugiperda* cells with this virus resulted in high level expression of the 52.4K NSs protein, even visible with Coomassie Blue in a polyacrylamide gel. Using antibodies raised against this product the NSs protein could be detected in infected *N. rustica* tissue both by ELISA and by Western analysis (R. Kormelink, unpublished results).

SEQUENCE INFORMATION ON THE L RNA SEGMENT

In an attempt to eludicate the sequence of the M RNA cDNA clones were prepared, using similar approaches as for the S RNA, and selected by Northern analysis. Thus a series of overlapping clones was obtained with an unambiguous restriction

map and corresponding to an RNA species of approximately 4.6 kilobases. Careful inspection of the Northern hybridization patterns revealed, however, that this series of clones were not specific for M RNA but were derived from a shortened version of the L RNA, migrating in denaturing (methylmercury) gels on the position of M RNA and co-replicating with the intact genome parts (data not shown). Determination of the nucleotide sequence revealed that this shortened form of L RNA is 4757 residues long and has complementary termini over a stretch of 60 to 65 nucleotides. Since this RNA contains the correct termini as present in full-length L RNA, its existence cannot be explained by fragmentation of L RNA upon isolation. Strikingly, this shortened L RNA version (which we propose to refer to as L' RNA) contains a long ORF in the vc strand, starting with a start codon at position 34 and extending to a UAA stopcodon at position 4519 (Fig. 5).

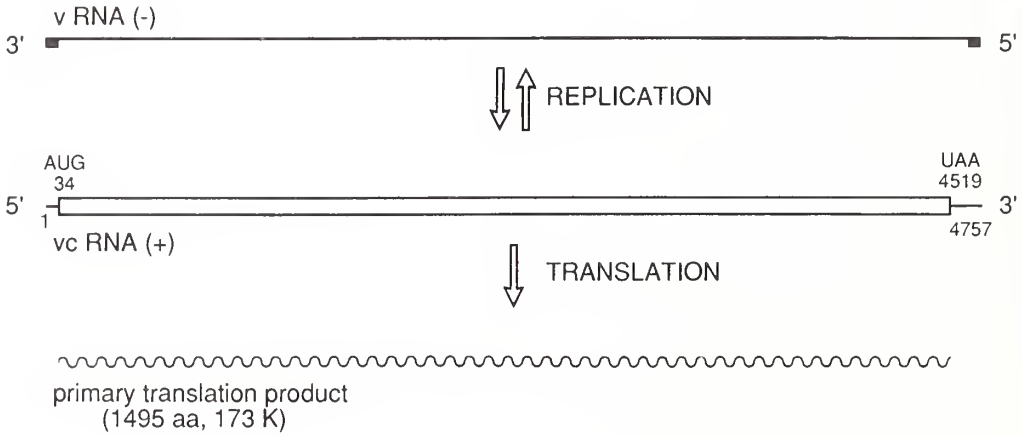

Figure 5. Genetic organization and expression of TSWV L' RNA. The viral strand (vRNA) is 4757 nucleotides long and is negative-stranded. The vc strand contains a single, long ORF corresponding with a primary translation product of 173K. L' RNA is a shortened derivative of L RNA, occurring in strain CNPH1, and co-replicating with the full-length genomic RNAs.

The coding capacity of this ORF would correspond with a primary translation product of 1495 amino acids and a molecular mass of 173K. Only within the N-terminal region this theoretical translation product exhibits some sequence homology to the L protein of Bunyamwera virus (Fig. 6), the only bunyavirus from which sequence information on the L RNA is available. The function of the L' RNA and its translation product has remained unknown sofar but the occurrence of smaller than genome-length RNAs may be characteristic for laboratory strains that have been maintained by mechanical transmission (see contribution of De O. Resende *et al.* in this volume). Nevertheless the sequence of the L' RNA, which is completely present in the full-length L RNA, demonstrates that TSWV is a negative-strand virus. Future work will concentrate on the determination of the sequences present in L RNA and absent in L' RNA.

TSWV IS A BUNYAVIRUS

The molecular data obtained and described above confirm previous statements, based on morphological data, that TSWV should by regarded as a member of the arthropod-borne Bunyaviridae, a large family of animal viruses. The nucleotide sequence data published elsewhere (De Haan *et al*, 1989, 1990) and discussed here provide the first firm proof that TSWV indeed belongs to this virus family. The sequence of L' RNA, a shortened, co-replicating version of the L RNA genomic segment, demonstrates that TSWV is a negative strand virus. The occurrence of this truncated RNA molecule has not been fully understood but may be linked to the partial defectiveness of the CNPH1 isolate due to maintainance by mechanical transmission. The genetic organization and ambisense expression strategy of S RNA are strikingly similar to those of the S RNAs from phleboviruses and uuku-viruses, two genera of the Bunyaviridae. The lack of significant sequence

Fig. 6. Alignment of the primary translation products of Bunyamwera L RNA (Elliott, 1989) and TSWV L' RNA, using the alignment program developed by the University of Wisconsin Genetics Computer Group (UWGCG). Top line, TSWV L protein sequence; bottom line, Bunyamwera virus L protein sequence.

homology, both on RNA and on protein level, indicates however that within the Bunyaviridae TSWV belongs to a distinct genus, which could be named phytophlebovirus or alternatively phythrivirus (siglum of phytos and thrips-transmitted).

ACKNOWLEDGEMENT

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REFERENCES

Blobel, B. 1980. Intracellular protein topogenesis. Proceedings of the national Academy of Sciences U.S.A. 77: 1496-1500.

De Haan, P., Wagemakers, L., Goldbach, R. & Peters, D. 1989a. Tomato spotted wilt virus, a new member of the Bunyaviridae? In Genetics and Pathogenicity of Negative Strand Viruses, pp. 287-290. Edited by D. Kolakofski & B.W.J. Mahy. Amsterdam: Elsevier Science Publishers B.V.

De Haan, P., Wagemakers, L., Peters, D. & Goldbach, R. 1989b. Molecular cloning and terminal sequence determination of the S and M RNA of tomato spotted wilt virus. Journal of General Virology 70: 3469-3473.

De Haan, P., Wagemakers, L., Peters, D. & Goldbach, R. 1990. The S RNA segment of tomato spotted wilt virus has an ambisense character. Journal of General Virology in press.

Emery, V.C. and Bishop, D.H.L. 1987. Characterization of Punta Toro S mRNA species and identification of an inverted complementary sequence in the intergenic region of Punta Toro phlebovirus ambisense S RNA that is involved in mRNA transcription termination. Virology 156: 1-11.

Elliott, R.M. 1989. Nucleotide sequence analysis of the large (L) genomic RNA segment of Bunyamwera virus, the prototype of the family Bunyaviridae. Virology 173: 426-436.

Ie, T.S. 1970. Tomato spotted wilt virus. Association of Applied Biologists Descriptions of Plant Viruses 39.

Ihara, T., Akashi., H. & Bishop, D.H.L. 1984. Novel coding strategy (ambisense genomic RNA) revealed by sequence analysis of Punta Toro phlebovirus S RNA. Virology 136: 293-306.

Matthews, R.E.F. 1982. Classification and nomenclature of viruses: Fourth report of the international committee on Taxonomy of Viruses. Intervirology 17: 1-200.

Milne, R.G. & Francki, R.I.B. 1984. Should tomato spotted wilt virus be considered as a possible member of the family Bunyaviridae? Intervirology 22: 72-76.

Mohamed, N.A., Randles, J.W. & Francki, R.I.B. 1973. Protein composition of tomato spotted wilt virus. Virology 56: 12-21.

Romanowski, V., Arsura, Y. & Bishop, D.H.L. 1985. Complete sequence of the S RNA of lymphocytic choriomeningitis (WE strain) compared to that of Pichinde arenavirus. Virus Research 3: 101-114.

Simons, J.F., Hellman, U. & Petterson, R.F. 1990. Uukuniemi virus S RNA segment: Ambisense coding strategy, packaging of complementary strands into virions and homology to members of the genus phlebovirus. Journal of Virology 64: 247-255.

Tas, P.W.L., Boerjan, M.L. & Peters, D. 1977. The structural proteins of tomato spotted wilt virus. Journal of General Virology 36: 267-279.

Verkleij, F.N. & Peters, D. 1983. Characterization of a defective form of tomato spotted wilt virus. Journal of General Virology 64: 677-686.

Van den Hurk, J., Tas, P.W.L. & Peters, D. 1977. The ribonucleic acid of tomato spotted wilt virus. Journal of General Virology 36: 81-91.

Von Heijne, G. 1983. Patterns of amino acids near signal sequence cleavage sites. European Journal of Biochemistry 133: 17-21.

Zuidema, D., Schouten, A., Usmany, M., Maule, A.J., Belsham G.J., Klinge-Roode E.C. Van Lent, J.W.M. & Vlak, J.M. 1990. Expression of cauliflower mosaic virus gene I using a novel polyhedrin-based baculovirus expression vector. Manuscript in preparation.

PHYSICAL MAP AND SEQUENCE INFORMATION OF THE S-RNA OF A BULGARIAN ISOLATE OF TSWV (TSWV-L3)

E. Maiss[1], L. Ivanova[2], E. Breyel[3], and G. Adam[3]

ABSTRACT

Purified virus cores extracted from complete tomato spotted wilt virus particles (TSWV) after Triton X-100 treatment were used to isolate total genomic RNA. Thes RNAs and gel purified S-RNA served as templates for the synthesis of complementary DNA. The generated cDNA molecules were blunt-end ligated into the HincII site of pT7T3 19U and transformed into E. coli. After colony filter hybridization with end labelled genomic TSWV-RNA, positive clones were selected. Size determination of approximately 100 clones revealed inserts ranging from 0.3 kb up to 2.2 kb. In hybridization experiments with northern blots of TSWV genomic RNA, specific clones to L, M and S RNA were detected. Clones specific to S RNA of TSWV were futher selected by cross hybridization with different dot-blotted clones from mini preparations. From these data and results of various restriction enzyme digestions a physical map was constructed for S RNA specific clones. The clones cover approximately 2.8 kb of the S-RNA. Sequencing of selected clones revealed an ambisense character of TSWV-L3 S RNA with two ORFs, the larger one located on the viral RNA, the smaller one on the complementary strand, encoding proteins of 52.4 kDa and 28.9 kDa, respectively. The 28.9 kDa protein was shown to be the capsid protein of TSWV-L3.

INTRODUCTION

TSWV causes economically important yield losses in a wide range of important crops all over the world. It differs from all other plant viruses in particle structure and genome organization. Transmission of the virus is exclusively facilitated by thrips species in a persistent manner. Because of these features TSWV represents a distinct virus group, the tomato spotted wilt virus group (Matthews, 1982).

Virions of TSWV are enveloped particles of about 80-110 nm diameter. At least four proteins could be found in the particle. The capsid protein of Mr 28 kDa, which is associated with the genomic RNAs, two or three glycoproteins and a large protein of 120 kDa. Genomic single-stranded RNAs of TSWV extending approximately 3200 (S RNA), 5400 (M RNA) and 8200 (L RNA) nucleotides. From these and other data it has been suggested to consider TSWV as a member of the Bunyaviridae (Milne and Francki, 1984; De Haan et al., 1989a).

To collect more information about the molceular biology of TSWV, the nucleotide sequence of the S RNA has been determined.

MATERIALS AND METHODS

Virus purification, cloning of cDNA and northern hybridization. Virus purification and core preparation was described by Adam et al (see this volume). Complementary DNA of total genomic and gel purified S RNA of TSWV-L3, a Bulgarian isolate of TSWV, was synthesized according to Gubler and Hoffman (1983) and blunt-end ligated into HincII cut pT7T3 19U phagemid vector (Pharmacia). Competent E. coli cells (strain NM 522) were transformed by the method of Hanahan (1983). Genomic RNAs of TSWV were denatured according to Carmichael and McMaster (1980), separated in 1% agarose gels, blotted onto nitrocellulose and hybridized with random primed ^{32}P labelled cDNA clones (Feinberg and Vogelstein, 1983a,b).

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1 Biologische Bundesanstalt für Land- und Forstwirtschaft, Institut für Biochemie, Messeweg 11-12; D-3300 Braunschweig F.R.G.

2 Central Laoratory of Genetic Engineering Agricultural Academy; 2232 Kostinbrod; Bulgaria.

3 Deutsche Sammlung für Mikroorganismen, Abteilung Pflanzenvirologie, c/o Biologische Bundesanstalt für Land- und Forstwirtschaft, Messeweg 11/12, D-3300 Braunschweig F.R.G.

Sequencing of cDNA clones. Single-stranded DNA templates of original or exonuclease III treated clones (Henikoff, 1984) were produced by infection of E. coli cells, harboring the plasmid of interest, with the helper phage M13K07 (Vieira and Messing, 1987). Sequencing reactions using the universal primer or T3 promoter specific primer were performed according to Sanger et al. (1977). Sequencing data were processed by the Sequence Analysis Software Package of the Genetics Computer Group (University of Wisconsin).

Construction of TSWV coat protein expression vector and production of the fusion protein. The insert of clone pTSWV-L3/308 was clonsed in frame into EcoRV/PstI cut pEX2 vector. The resulting expression plasmid pEXcpTSWV-L3 was transformed into E. coli pop 2136 cells. Production of the truncated -galactosidase-TSWV fusion protein was essentially as described by Maiss et al. (1990).

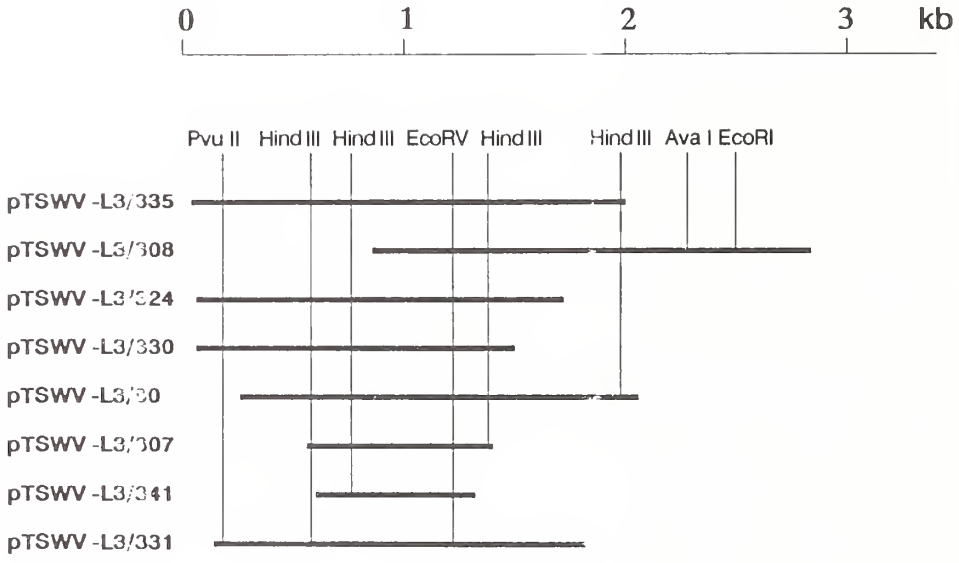

Fig. 1: Physical map of cDNA clones specific to S RNA of TSWV-L3 (Bulgarian isolate).

RESULTS AND DISCUSSION

Clone pTSWV-L3/335 specific to S RNA of TSWV was detected by northern hybridization to denatured genomic TSWV-rNAs. By cross hybridization of this clone and subsequent restriction enzyme digestions, S RNA specific clones were further selected and a physical map was constructed (Fig. 1).

Sequence determination of both cDNA strands was carried out mainly from clones pTSWV-L3/335 and pTSWV-L3/308. The assembled sequence revealed 2837 nucleotides. Comparison with the terminal sequences of TSWV isolate CPNH1 (De Haan et al., 1989b) revealed a 100% identity starting at position 42 at the 5'-end (Fig. 2). No sequence homologies were found at the 3'-end.

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Position        42

TSWV-CPNH1    5'-TAGAAAATCACAATACTGTAATAAGAACAC - 3'
TSWV-L3/335   5'-TAGAAAATCACAATACTGTAATAAGAACAC - 3'

Position         1
```

Fig. 2. Comparison of TSWV-CPNH1 5'-end with pTSWV-L3/335 5'-end

The S RNA of TSWV-L3 displays only two significant ORFs; the larger one is encoded on the viral RNA strand, the other one on the complementary strand. Both ORFs are separated by a non-coding region of 586 nucleotides. This region is very rich in adenosine (37.5%) and thymidine residues (41.5%) and is able to form a highly stable secondary structure by internal base pairing of the thymidine rich stretch and the inverted complementary adenosine region.

The ORF of the viral RNA is 1401 nucleotides in length and can encode a protein with 467 amino acids of Mr 52.4 kDa. Five possible glycosylation sites (NXT/S; Doolittle, 1986) were found in the amino acid sequence. No homologies could be observed to any protein in the GenBank (June, 1989, Release 60.0) and the EMBL Data Bank (Release 19.0. May, 1989).

The ORF of the complementary S RNA strand is 774 nucleotides long and encodes a protein of 258 amino acid corresponding to Mr of 28.9 kDa. No glycosylation sites were detectable.

Data base searching for homologies with other proteins did not reveal any significant identities. Because of tis molecular weight we assumed, that this protein is the capsid protein of TSWV. To prove this hypothesis, the insert of pTSWV-L3/308 was subcloned into the expression vector pEX2, to give pEXcpTSWV-L3. The produced fusion protein was separated in 10% polyacrylamide gels and after Western blotting immuno-stained with antibodies specific to the capsid protein. The molecular weight of the expected fusion protein was calculated to be 76 kDa. In Coomassie-stained gels a protein band of approximately 78 kDa was observed. This band reacts specifically with the antibodies against the capsid protein. Therefore it was evident that the 28.9 kDa protein encoded on the complementary strand of the S RNA is the capsid protein of TSWV.

TSWV S RNA encoded a non-structural protein and the capsid protein in an ambisense character. Similar genome expression is observed by the S RNAs of viruses belonging to the Phlebo- and Uukuviruses in the family Bunyaviridae. Viruses of both groups expressing the coat protein and in addition a non-structural protein, called NSs in an ambisense character like TSWV. Because of the lack of any significant homologies of the non-structural proteins with the 52.4 kDa, TSWV should be placed in a separate group within the Bunyaviridae.

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REFERENCES

Carmichael, G. G. and G. K. McMaster. 1980. The analysis of nucleic acids in gels using glyoxal and acridine orange. Methods in Enzymology 65:380-391.

De Haan, P., L. Wagemakers, R. Goldbach, and others. 1989a. Tomato spotted wilt virus, a new member of the Bunyaviridae? In Genetics and Pathogenicity of Negative Strand Viruses, pp. 287-290. Ed.: D. Kolakofski and B. W. J. Mahy. Elsevier Science Publishers B.V., Amsterdam.

De Haan, p., L. Wagemakers, D. Peters, and others. 1989b. Molecular cloning and terminal sequence determination of the S and M RNAs of tomato spotted wilt virus. J. Gen. Virol. 70:3469-3473.

Doolittle, R. F. 1986. In On URFs and ORFs. Oxford: Oxford University Press.

Feinberg, A. P. and B. Vogelstein. 1983a. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 132:6-13.

Geinberg, A. P. and B. Vogelstein. 1983b. A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Addendum. Anal. Biochem. 137:266-267.

Gubler, U., and B. J. Hoffman. 1983. A simple and efficient method for generating cDNA libraries. Gene 25:263-269.

Henikoff, S. 1984. Unidirectional digestion with exonuclease III creates targeted breakpoints for DNA sequencing. Gene 28:351-359.

Hanahan, D. 1983. Studies on transformation of Escherichia coli with plasmids. Journal of Molecular Biology 166:557-580.

Maiss, E., U. Timpe, and R. Casper. 1990. Expression of the coat protein gene of plum pox virus in Escherichia coli. Arch. Phytopathol. Pflanzenschutz, Berlin 26: (in press).

Matthews, R. E. F. 1982. Classification and nomenclature of viruses: Fourth report of the International Committee on Taxonomy and Viruses. Intervirology 17:1-200.

Milne, R. G., and R. I. B. Francki. 1984. Should tomato spotted wilt virus be considered as a possible member of the family Bunyaviridae? Intervirology 22:72-76.

Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. Proceedings of the National Academy of Science, U.S.A. 74:5463-5467.

Vierra, J. and J. Messing. 1987. Production of single-stranded plasmid DNA. Methods in Enzymology 153:3-11.

DEFECTIVE ISOLATES OF TOMATO SPOTTED WILT VIRUS

R. de O. Resende, E.W. Kitajima, A.C. de Avila, R.W. Goldbach & D. Peters

Department of Virology, Agricultural University, Binnenhave 11, P.O. Box 8045, 6700EM Wageningen, the Netherlands.

ABSTRACT

Defective isolates of TSWV may arise by mechanical inoculation in repeated transfers. They are characterized by the inability to form functional membrane proteins and as a consequence by the failure to form complete virus particles. Serial passages of some isolates were made to induce and to isolate defective forms, but not all procedures used were successful. The development of defective isolates during transfers of virus isolates can be monitored by ELISA techniques using anti-N and anti-G monoclonals and using gold labelled anti-N and -G monoclonals in dip preparations.

INTRODUCTION

It is common practice to maintain TSWV isolates in the laboratory by mechanical transmission. The selective pressure to which the virus is exposed under this condition will certainly differ from that applied to that prevailing in the field.

It has been reported (Ie, 1982) that after repeatedly mechanical transfers of a number of TSWV isolates the characteristic enveloped virus particles could not any more detected in the cells of infected plants. In stead only dense amorphous, more or less rugged masses are found in the cytoplasm of infected cells (Fig. 1). These masses, consisting of electron dense structures with a lattice periodicity of about 5 nm, are not bounded by a membrane and are usually somewhat larger in size than the mature virus particles. These masses are scattered in the cytoplasm among the ribosomes close to the endoplasmic cisternae where the virus particles accumulate. These structures were called 'dense strands' by Kitajima (1965) and amorphous darkly stained material' by Milne (1970). On repeated transfers of isolates by sap inoculation, these masses tend to increase in number whereas the virus particles become less frequent (Kitajima et al., in preparation), or eventually disappear completely (Ie, 1982).

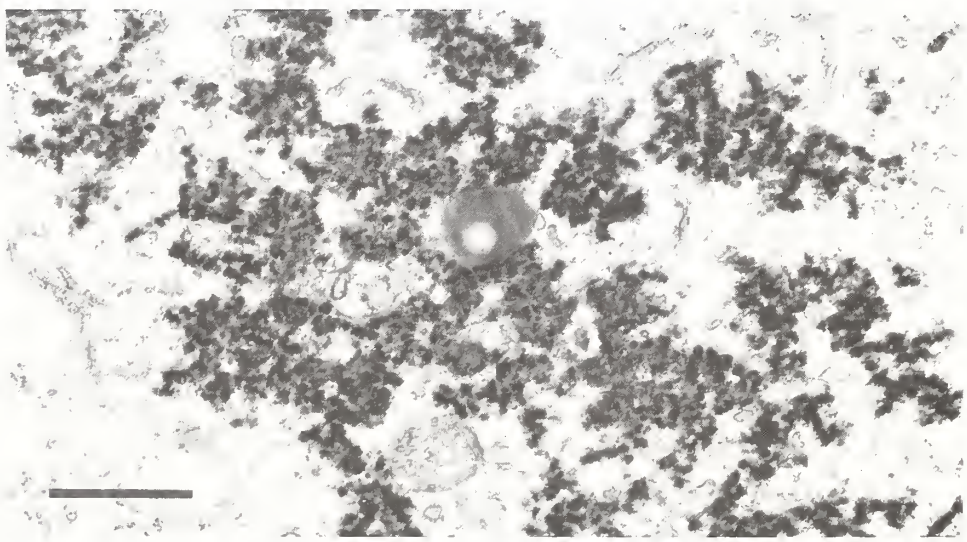

Fig. 1. Mesophyll cell of Nicotiana tabacum cv. Samsun NN infected with tomato spotted wilt virus isolate P/S-NN. The cytoplasma contains only amorphous masses. (Bar represents 1 um).

CHARACTERIZATION OF THE DEFECTIVE ISOLATES

The absence of complete particles in cells and the transmission of a component that was infectious suggested that the virus had become defective. In ELISA studies, a reaction was only found in extracts from plants infected both with isolates producing virus particles and with isolates producing dense masses only with anti-N immunoglobulins. A positive reaction with the anti-G-immunoglobulins was only found when plants were infected with isolates which were still able to produce the characteristic particles (Verkleij & Peters, 1983). The defective isolates did not produce any detectable amount of viral membrane proteins. The possibility that the viral membranes were synthesized, but disappeared thereafter instead of being used for assembly of the virus particles was studied in a kinetic study. It was evident that the amount of N protein increased with time for both types of isolates, but that the G protein could only be detected in the material infected with virus particles producing isolates (Verkleij & Peters, 1983).

Electrophoretic analysis of the RNA from both types of isolates showed that both had a tripartite genome. The L and S RNA had apparently not changed in size, but the M RNA of the defective isolates migrated more rapidly than that of normal isolates. The M RNA segment of the three different defective isolates studied appeared to be 600 to 900 bases smaller (Verkleij & Peters, 1983). The defective isolates generated by Ie (1982) should therefore be considered as isolates with deletions in their M RNA. The nature of the deletions and the process by which they arose can, unfortunately, not be studied in more detail since the original virus isolates were not stored properly and were lost during the repeated transfers.

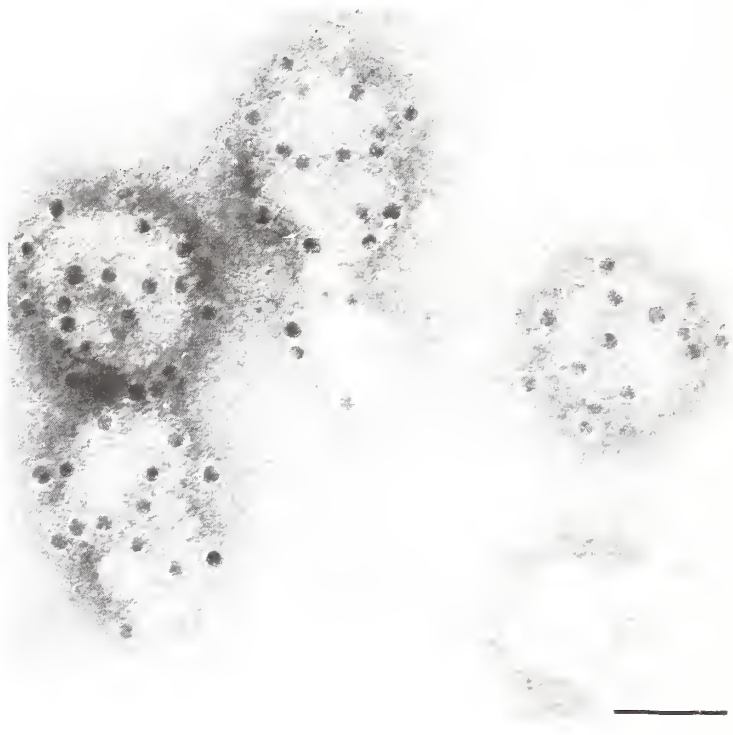

Fig. 2. Micrograph of a dip preparation made from Nicotiana rustica plants infected with a defective form of the chrysanthemum isolate of tomato spotted wilt virus (H4). The preparations were treated with gold labelled anti-nucleocapsid protein immunoglobulins. The structures, coined "cotton balls" reacting with the labelled gold, are believed to be dense masses. (Bar represents 100nm).

STUDIES TO GENERATE NEW DEFECTIVE FORMS.

An attempt was made to induce and subsequently to isolate defective forms of our laboratory strain CNPH1 of which the S RNA segment has been completely sequenced (de Haan et al., 1990A) and the M and L RNA partially (de Haan et al., 1990b). Electron microscopical studies have shown that considerable amounts of dense masses occur simultaneously with intact virus particles in the cells infected with this isolate. A large number of local lesions were sequentially transferred on Nicotiana tabacum cv 'Samsun NN'. Three types of local lesions occurred after inoculation of which an equal number was used to produce a new generation of local lesions. After four transfers, 18 local lines were analyzed after propagation on Nicotiana rustica by ELISA. Since all local lesions lines reacted with the anti-N-monoclonal as well as with the anti-G-monoclonals (Huguenot et al., 1990) it was concluded that completely defective isolates were not isolated by this repeated local lesion transfer. Hence, it is concluded that although CNPH1 represents a partially defective isolate, no completely defective lines could be isolated. This suggests that the amorphous masses (defective particles) and intact particles are produced according to a strictly maintained balance.

It deserves to be noted that although three different types of local lesions could be distinguished, all local lesion lines produced almost identical symptoms after back inoculation on N. rustica.

SPONTANEOUSLY OCCURRING DEFECTIVE ISOLATES

In the serological studies made with a Dutch isolate from chrysanthemum, and a Brazilian isolate from tomato, B13, it was noticed that the reaction with the anti-G monoclonals decreased considerably after a few transfers over N. rustica. The development of these isolates was further studied in serial passages over different hosts and ELISA using anti-N and anti-G monoclonal antibodies. In dip preparations structures, referred to as "cotton balls" (Fig. 1), reacted with immunogold labelled anti-N monoclonals. These structures were believed to be identical to the dense masses in the cytoplasm and dissociated to some extent during preparation of the specimen. Anti-G monoclonals did not react with these structures. The isolate from chrysanthemum was serially transferred in two lines over different hosts (Fig. 3). Passage over N. rustica resulted in a partial revovery of the original reaction to anti-G monoclonals in ELISA (Fig. 4). These results indicate that the amount of G protein has increased and that a part of infectious material consists of complete virus particles. This could be confirmed by electron microscopy of both thin sections and dip preparations. A complete defective

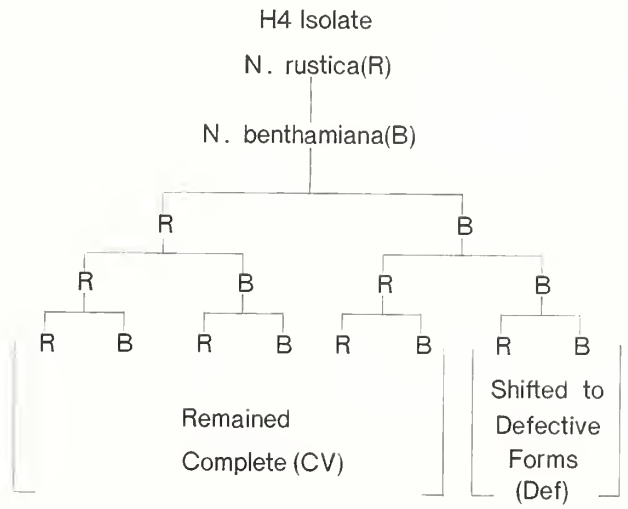

Fig. 3. Flow diagram to induce and to isolate defective forms of a chrysanthemum (H4) of tomato spotted wilt virus.

form was found when the chrysanthemum isolate was passaged over Nicothiana benthamiana as verified by ELISA (Fig. 4) and electron microscopy (results not shown). However it should be noted that there is still a rudimentary reaction with MAb-G3 (Fig. 4). A slow sedimenting fraction in a sucrose gradient on which the nucleocapsid fractions were analysed, reacted also weakly with MAb-G3 and MAb-G1 in ELISA (results not shown). These positive reactions indicate that a protein which react with MAb-G monoclonals is produced in small amounts during the infection. At this moment it is not known whether this isolate is indeed completely defective or that it contains still an undetectable number of intact particles. The virtually defective form isolated induces a very severe necrosis on both tomato and N. rustica; the infected plants even die within two weeks. The inoculum of the partially recovered isolate, on the other hand, produces moderate symptoms in the first systemically infected leaves whereas only faint symptoms occur on the leaves which develop later. RNA analysis showed that the defective isolate contains three RNA segments, being apparently of the same size as those of normal TSWV particles. The partially recovered isolate contained four RNA segments; the S, M and L RNA's were similar in size to those of the defective isolate. However, the fourth segment migrated slower than the S RNA segment.

The Brazilian isolate (B13) was serially transferred in three different lines over various host plant species (Fig. 5). Three variants were obtained; one (L1), which was transferred five times over N. rustica, produced a severe necrotic reaction on this host, the second one (L2) produced severe symptoms, whereas the third isolate (L3) passaged three time over Gomphrena globosa

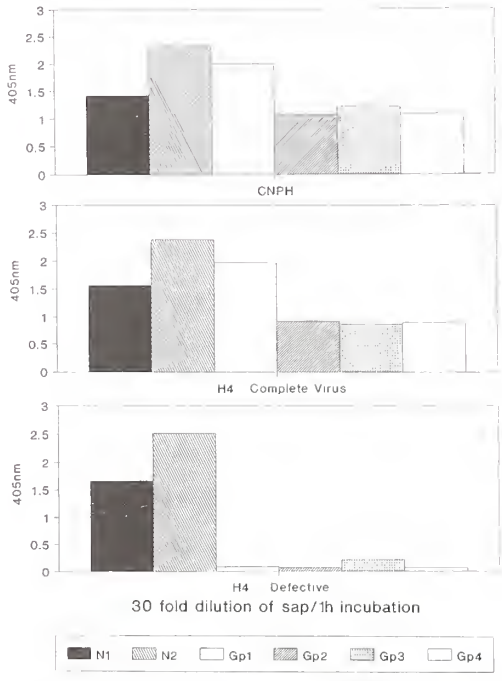

Fig. 4. Serological reaction of two isolates of the Dutch chrysanthemum H4 TSWV isolate with monoclonal antibodies to the N and G proteins after serial passage over different hosts. (R=isolate after passage over N. rustica, CV-partially recovered isolate, Def=defective isolate, see Fig. 3).

plants, a mild mottling. These isolates were analyzed by ELISA. The three variants produced similar amounts of nucleocapsid protein, but the amount of G protein increased with the severity of the symptoms (Fig. 6). Electron microscopic studies revealed that with the increasing amounts of G protein more particles could be detected in cells infected with these isolates. With respect to symptom expression it can be concluded that the amount of G protein found in the respective isolates seems not to determine the severity of symptoms.

DISCUSSION

The results obtained by Ie (1982) and Verkleij & Peters (1983) have shown that defective isolates may arise by deletions in the M RNA. Since complete particles were not assembled it was concluded that at least the membrane protein G1 was coded for by the M RNA. The defective isolate obtained from the Dutch chrysanthemum isolate (H4) containing an M RNA segment of wild type length, may have captured a stop codon or other point mutations that may prevent the synthesis of functional membrane proteins.

It is striking that though no functional membrane proteins are formed, the M RNA, either deleted or still having its wild type length, is not lost in the successive replications of the defective forms. This may mean that in addition to coding for the membrane proteins, M RNA specifies another protein which is still essential even for the replication of the defective forms.

Kitajima et al. (in preparation) showed that the amount of dense masses increased gradually with the number of mechanical transfers made. We were not able to isolate by repeated transfers of local lesions defective forms of the CNPH isolate, which produces a huge amount of dense masses in the cytoplasm. This failure may indicate that not all dense masses produced consist of nucleocapsid material of defective variants. It is possible that they are also formed in situations in which the assembly of virus particles is out of balance due to a much higher production of nucleocapsids than of membrane proteins.

Alternatively, accumulation of dense masses may also suggest that defective variants may occur, which can not replicate without complementation of certain functions by a complete variant. The accumulation of dense masses in the cytoplasm will then be a property analogue to the enriched replication of defective interfering particles in other virus sytems. Contrary to the defective interfering particles of other viruses which usually maintain the original particle morphology, our assumed TSWV variant will then be a defective interfering variant which has lost the ability to form complete virus particles (Huang & Baltimore, 1977).

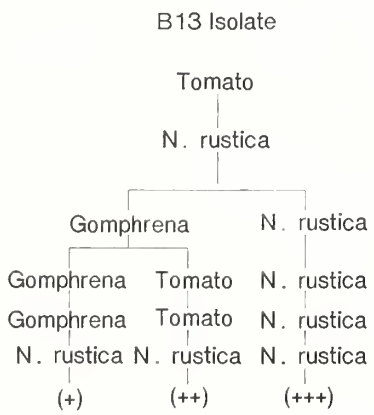

Fig. 5. Flow diagram to induce and to isolate defective forms of a Brazilian tomato spotted wilt isolate (B13).

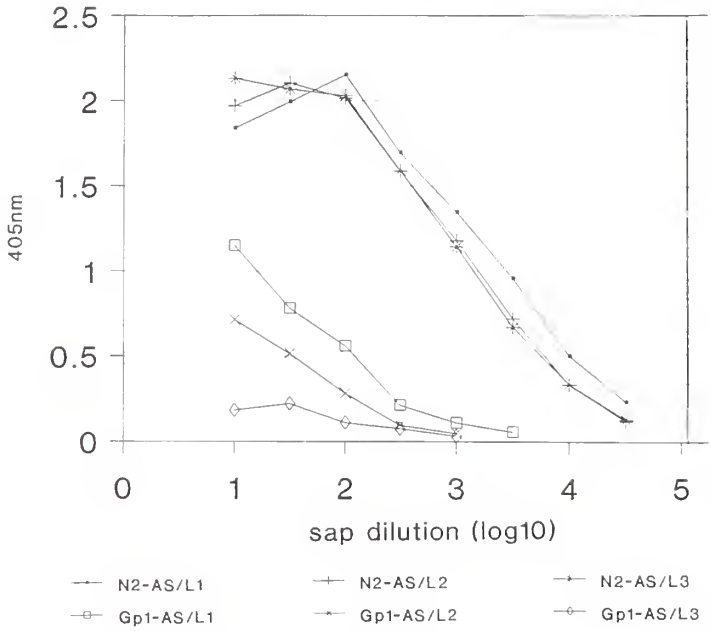

Fig. 6. Serological reactions of three isolates of B13, a Brazilian isolate of tomato spotted wilt virus, using anti-nucleocapsid protein (N2) and (Gp1) monoclonal antibodies. L1 induces a strong necrosis, L2 severe symptoms and L3 a weak mottling on Nicotiana rustica.

REFERENCES

de Haan, P., Wagemakers, L., Peters, D. & Goldbach, R. (1990a). The S RNA segment of tomato spotted wilt virus has an ambisense character. J. Gen. Virology (in press).

de Haan, P., Kormelink, R., Peters, D. & Goldbach, R. (1990b). Genetic organization and expression of the tomato spotted wilt virus genome. (These proceedings).

Huang, A.S., & Baltimore, D. (1977). Defective interfering animal viruses. In: Comprehensive Virology (Eds. H. Fraenkel-Conrat & R.R. Wagner). Plenum Press, New York, Vol. 10: pp 73-116.

Huguenot, C., van den Dobbelsteen, G., de Haan, P., Wagemakers, C.A.M., Drost, G.A., Osterhaus, A.D.M.E. & Peters, D. (1990). Detection of spotted wilt virus using monoclonal antibodies and riboprobes. Arch. Virology 110:47-62.

Ie, T.S. (1971). Electron microscopy of developmental stages of tomato spotted wilt virus in plant cells. Virology 43: 468-479.

Ie, T.S. (1982). A sap-transmissible, defective form of tomato spotted wilt virus. J. Gen. Virology 59: 387-391.

Kitajima, E.W. (1965). Electron microscopic of viracabeca (Brazilian tomato spotted wilt virus) within the host cell. Virology 26: 89-99.

Milne, R.G. (1970). An electron microscope study of tomato spotted wilt virus in sections of infected cells and in negative stain preparations. J. Gen. Virology 6; 267-276.

Verkleij, F.N. & Peters, D. (1983). Characterization of a defective form of tomato spotted wilt virus. J. Gen. Virology 64: 677-686.

THE OCCURRENCE OF YELLOW SPOT VIRUS, A MEMBER OF TOMATO SPOTTED WILT VIRUS GROUP, ON PEANUT (Arachis hypogaea L.) IN INDIA*.

D.V.R. Reddy, M.R. Sudarshana, A.S. Ratna, A.S. Reddy, P.W. Amin, I.K. Kumar, and A.K. Murthy

ABSTRACT

A virus that induced yellow leaf spots which later coalesced and became necrotic, was isolated from peanut (Arachis hypogaea) in India and named peanut yellow spot virus (PYSV). PYSV was sap-transmissible to eleven species of Chenopodiaceae, Leguminosae, and Solanaceae. The virus induced local lesions in all the hosts. V. unguiculata was a good local lesion host. The infection in Pisum sativum, Vigna radiata and V. unguiculata became systemic when they were maintained at temperatures between 20 and 30°C. In thin sections of peanut leaves spherical membrane-bound particles of 70 to 100 nm in diameter were observed to occur in clusters. Purified virus preparations contained five polypeptides of 90000, 54000, 44000, 40000 and 31000 dalton polypeptides. When virus particles were treated with Nonidet P 40, the polypeptides were removed with the exception of that of 31000 daltons. In both ELISA and western blots, PYSV did not react with antisera to two tomato spotted wilt virus isolates. On the basis of these properties, PYSV is regarded as a previously undescribed virus belonging to the tomato spotted wilt virus group.

INTRODUCTION

Peanut (Arachis hypogaea L.) is affected by several virus diseases in India (Reddy, In press). One virus disease which is characterized by yellow leaf spots which later coalesce and become necrotic was reported in 1978 from several locations (ICRISAT, 1978). The causal virus was named "peanut yellow spot virus" (PYSV). In thin sections of peanut leaflets, membrane-bound virus particles of 70 to 100 nm, similar in morphology to tomato spotted wilt virus (TSWV), were observed. But in host range and symptoms, the virus differed from the TSWV reported from India by Ghanekar et al. (1979). In disease surveys PYSV was found to be widely distributed in India. A similar disease was also observed in peanut in Thailand (Wongkaew et al., 1985).

We report here host range, symptomatology, purification, serological relationships and protein analysis of the PYSV.

MATERIALS AND METHODS

Virus culture and maintenance

Peanut leaflets showing small chlorotic spots (initial symptoms) were collected from the field. Extracts prepared in

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Reddy is Principal Plant Virologist, and Sudarshana, Ratna and Reddy are Research Associates in the Virology Unit, Legumes Program, ICRISAT; Amin is a Senior Scientist in Asian Grain Legumes Network, ICRISAT; Kumar was deceased. Murthy is engineer in Electron Microscope Unit, ICRISAT.

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0.05 M phosphate buffer, pH 7.0, containing 0.075% thioglycerol (PBT) were inoculated onto peanut plants (cv. TMV 2). The virus later isolated from these plants was maintained either in peanut or in Vigna radiata (cv. HY 45).

Assay host:

Since the virus consistently produced distinct chlorotic local lesions on Vigna unguiculata (cowpea, cv. C 152), this cultivar was chosen as a local lesion assay host.

Host range studies:

Extracts from 0.5 - 1 g of young peanut leaflets showing initial symptoms were inoculated onto six plants of each of 17 test species and these were maintained in a glasshouse for 45 days at 20-30°C. Extracts from inoculated and non-inoculated leaves of each test plants were checked for infection by sap inoculation onto V. unguiculata, and by protein - A coating (PAC) enzyme-linked immunosorbent assay (ELISA) (Hobbs et al. 1987) using a homologous antiserum.

Properties of the virus in sap:

The physical properties of the virus were determined in extracts from peanut leaflets prepared in PBT. For determining thermal inactivation point (TIP), one ml aliquots of sap diluted to 10^{-1} were heated at a range of temperatures from 40 to 75°C in a water bath for 10 minutes. Longevity in vitro (LIV) was determined using a 10^{-1} dilution incubated at 25°C for up to 2 days. The dilution end point (DEP) was determined by diluting the sap from 10^{-1} to 10^{-4}.

Electron microscopy:

Portions of peanut leaflets showing initial symptoms were prefixed overnight in 3% glutaraldehyde in 0.1 M phosphate buffer, pH 7.2, and then washed thrice in the buffer. The pieces were then post-fixed in 2% aqueous osmium tetroxide for 6 hours, followed by three washings in distilled water. Dehydration was done in a graded series of acetone, and the pieces were then embedded in Spurr's medium (Spurr, 1969). Ultrathin sections were cut and stained in uranyl acetate and lead citrate, then examined in an electron microscope (Philips 201 C).

Virus purification:

All purification steps were carried out at, or near to, 4°C. The procedure was similar to that developed for purification of TSWV from peanut (M.R. Sudarshana, I.K. Kumar, A.S. Ratna, R. Rajeshwari, and D.V.R. Reddy, in preparation). Peanut leaflets showing initial symptoms of light green or pale yellow spots (green areas of leaflets removed) were frozen at -80°C. Frozen leaflets were triturated in a blender in an extractant containing 0.02 M phosphate buffer, pH 7.5, containing 0.01 M diethyl- dithiocarbamate and 0.02% monothioglycerol; at a rate of 4 ml for each gram of tissue. The homogenate was filtered through two layers of cheese-cloth and subjected to 10000 rpm for 20 minutes. The resulting pellet was suspended (1 ml per gram tissue) in 0.01 M phosphate buffer, pH 7.5, containing 0.01 M sodium sulfite (PBSS), stirred for 1 hour at 4°C and clarified at 10000 rpm for 10 minutes. The supernatant was subjected to rate zonal density gradient centrifugation for 1 hour at 20000 rpm in a Beckman SW28 rotor in gradients made of 8.0 ml each of 20, 30, 40% sucrose, and 10 ml of 50% sucrose

(w/v) in PBSS. A light scattering zone at a height of 4.3-5.5 cm was collected, diluted in PBSS and centrifuged at 30000 rpm for 1.5 hours in a Sorvall T865 rotor. The resulting pellets were suspended in PBSS and centrifuged at 10000 rpm for 5 minutes. The supernatant was subjected to another cycle of rate zonal gradient centrifugation. The major light scattering zone (4.3-5.5 cm height) was collected, diluted in PBSS, and pelleted.

Preparation of nucleocapsids:

Purified virus from 100 gram tissue was suspended in 10 ml of PBSS and Nonidet P-40 was added to give a final concentration of 1%. After stirring for 15 minutes it was subjected to rate zonal centrifugation in a Beckman SW 28 rotor as described above. A light scattering zone which appeared between 4.0 to 4.5 cm height was drawn, diluted in PBSS, and centrifuged at 30000 rpm for 1.5 hours in a Beckman R 40 rotor.

Serology:

Purified virus obtained from 50 gram tissue was suspended in 1.0 ml of a 0.01 M phosphate buffer, pH 7.0, containing 0.85% NaCl, and emulsified with 1.0 ml of Freund's incomplete adjuvant. Emulsion was injected intramuscularly into the hind leg of a New Zealand White inbred rabbit at three different sites. Five such injections were given at weekly intervals. Bleeding began 1 week after the last injection, and was carried out at weekly intervals. Serum was lyophilized and stored at -20^{o}C.

For testing serological relationships a protein A coating method of ELISA (PAC-ELISA) was used (Hobbs et al. 1987). Protein A at 1 ng/ml concentration, prepared in 0.05 M sodium carbonate buffer, pH 9.6, was used to coat wells of microtitre plates. Healthy and infected leaf extracts were prepared in an antigen buffer (Clark and Adams, 1977). Antisera were cross-adsorbed with healthy peanut leaf extracts (Hobbs et al. 1987). Anti-rabbit Fc-specific immunoglobulins from antiserum produced in goats (Cappel Laboratories Inc., Pennsylvania) were conjugated to penicillinase (B-lactamase) and used at 1:10,000 dilution (Sudarshana and Reddy, 1989). Sodium salt of penicillin-G was used as a substrate. Absorbance values were recorded at 620 nm in a Titertek Multiscan MCC ELISA plate reader for three replications of each sample.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE):

SDS-PAGE analysis of proteins of the purified virus was done according to the procedure of Laemmli (1970). Purified virus samples were suspended in a sample buffer containing 0.0625 M Tris-HCl, pH 6.8, 1% SDS, 15% glycerol, 0.5% 2-mercaptoethanol, and 0.001% bromophenol blue, and boiled for 5 minutes prior to loading. Samples were electrophoresed with markers at 35 V for 15 hours. Gels were stained with silver nitrate as follows. The gel was first fixed in a solution containing 50% methanol and 12% acetic acid for 30 minutes, then given three washes in distilled water, allowing 20 minutes for each wash. It was then treated for 30 minutes with 0.0005% dithiothreitol (DTT) in distilled water (a stock solution of 0.05% DTT was diluted to 1:100 before use). The DTT was then removed, and the gel treated with 0.2% silver nitrate, prepared in distilled water, for 30 minutes. The gel was then rapidly rinsed in distilled water and immersed in a developer (containing 3% sodium carbonate and 0.05% formaldehyde prepared in distilled water)

until the polypeptides were clearly visible, which usually took about 10 minutes.

The following protein markers from Bio-rad Laboratories were used: Phosphorylase B (92500), bovine serum albumin (66200), ovalbumin (45000), carbonic anhydrase (31000) and soybean tripsin inhibitor (21500). Molecular weights were determined from linear scans of gels on a LKB Ultroscan XL densitometer.

Electro-blot immunoassay:

Purified virus was subjected to SDS-polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane (Schleicher and Schull BA 85, 0.45 um pore size) utilizing a semi-dry transfer unit (TE 70 Semiphor, Hoefer Scientific Instruments, California). Nitrocellulose membranes presoaked in 0.192 M glycine, 0.025 M tris, pH 8.3, containing 0.0013 M SDS and 20% methanol (Towbin et al. 1979) were used and transblotting was done at 15 V for 1 hour. After transfer of proteins the membrane was incubated while shaking, for 2 hours in a solution of 5% (w/v) spray-dried milk (SDM, "Everyday" by Nestle) in tris-buffered saline (TBS, 0.02 M Tris-HCl, 0.5 M NaCl, pH 7.5). The membrane was then incubated for 1 hour in a 1/500 dilution of antisera in TBS with 0.05% tween-20 (TTBS) containing 5% SDM (TTBS-SDM). After washing three times for 10 minutes each in TTBS, the membrane was incubated for 1 hour in horseradish peroxidase-labelled anti-rabbit Fc-specific immunoglobulins, produced in goats, and prepared in TTBS-SDM. After the membrane was washed three times in TTBS, it was treated with a TMB membrane peroxidase substrate and an enhancer (Kirkegaard and Perry Laboratories, Maryland). Color (blue) development was recorded visually. The membrane was washed with distilled water, air-dried and photographed immediately.

RESULTS

Symptomatology and host range:

Initial symptoms in mechanically inoculated plants appeared as chlorotic spots, which enlarged and became pale yellow. As leaflets aged, spots became dark yellow and ultimately necrotic (Fig. 1). Coalescence of adjacent lesions sometimes led to death of leaflets.

Eleven of 17 plants tested were infected (Table 1). In all the hosts the virus induced local lesions (Fig. 2). Systemic infection in Pisum sativum, Vigna radiata and V. unguiculata occurred only when ambient temperatures were between 20 and 30°C. The following hosts were not infected by the virus: Brassica campestris, Cyamopsis tetragonolobus, Glycine max (cv. Bragg), Lycopersicum esculentum (cvs. Marglobe, Pusa Ruby), Nicandra physaloides and Solanum melongena.

Properties of the Virus in sap:

The DEP was between 10^{-2} and 10^{-3}, the TIP was between 45° and 50°C, and LIV was for 5 hours at 25°C.

Electron microscopy:

Spherical membrane-bound particles, ranging from 70 to 100 nm in diameter, were observed in the cytoplasm (Fig. 3). Numerous clusters of enveloped virus particles were also observed in cytoplasm.

Virus purification:

Preparations of purified virus were pale green. The purification method adopted yielded intact membrane-bound particles of 70-80 nm in diameter. Scattered membranous host contaminants were also present (Fig. 4).

Serology:

Cross-adsorption with healthy peanut leaflet extracts was necessary to avoid non-specific reaction presumably due to presence of antibodies to host material. In PAC-ELISA, PYSV reacted with homologous antiserum. It did not react with antisera to two TSWV isolates (Table 2).

SDS-PAGE:

Five major proteins of molecular weights 90000, 54000, 46000, 40000 and 31000 daltons (average of five determinations) were consistently resolved in 10% acrylamide gels. Several minor polypeptides, which varied from sample to sample, were also observed. These proteins were probably derived from host material.

Following treatment with Nonidet-40, the major polypeptide retained was that of 31000 daltons, while low proportions of 54000 and 40000 dalton polypeptides were also observed.

Electro-blot immunoassay:

The proteins separated by SDS-PAGE, were transferred to nitrocellulose by electrophoresis, and tested by a homologous and by two TSWV antisera. Using the homologous antiserum, all the five viral polypeptides could be detected. No reaction was observed with any of the polypeptides when TSWV antisera were used.

DISCUSSION

From the above results it is evident that PYSV resembles TSWV in particle morphology, polypeptide composition and physical properties in crude plant sap. Both viruses are transmitted by thrips (Amin and Mohammad, 1980). In host range studies, PYSV produced local lesions on Chenopodium amaranticolor, C. quinoa, Petunia hybrida, Phaseolus vulgaris, and Vigna unguiculata. However when TSWV and PYSV were assayed under similar conditions on V. unguiculata, PYSV took 9-12 days to produce lesions as compared with 4-5 days for TSWV. Also the lesions produced by PYSV were uniformly chlorotic and surrounded by a halo, whereas those produced by TSWV were of concentric chlorotic rings. This feature can be used to distinguish PYSV from TSWV. Unlike PYSV, TSWV infects Brassica campestris, Cyamopsis tetragonoloba, Glycine max, Lycopersicum esculentum, Nicandra physaloides and Solanum melongena (Reddy and Wightman, 1988). While TSWV induces systemic infection in Datura stramonium, Nicotiana benthamiana, and N. glutinosa, PYSV induces only local lesions.

In ELISA tests PYSV was shown to be serologically distinct from two TSWV isolates infecting peanuts, while none of the PYSV polypeptides reacted with the two TSWV antisera in electro-blot immunoassay.

In the morphology of virus particles, and in the presence of clusters of enveloped virus particles, PYSV closely resembled

TSWV (Francki and Grivell 1970; Ie, 1982).

The molecular weights of PYSV polypeptides differed from those of TSWV as reported by Milne and Francki (1984). The 54000 dalton polypeptide is considered to be envelope glycoprotein G1 and 46000 and 40000 polypeptides are considered to be glycoproteins G2. Evidence was presented to show that NP 40 treatment removed all major polypeptides except the 31000 dalton polypeptide. Thus, as in TSWV (Mohamed et al. 1973; Tas et al. 1977), this polypeptide is considered to be associated with the nucleocapsid.

Since TSWV is known to resemble members of the family bunyaviridae (Milne and Francki, 1984; de Haan et al. 1989) it is appropriate to group TSWV and any similar virus in the family "Phytobunyaviridae". To our knowledge, TSWV has been the sole member of this group, but based on the data presented in this paper, we consider that PYSV could be regarded as a distinct member of the "Phytobunyaviridae".

We are currently analyzing nucleic acids of PYSV and extent of homology between PYSV and TSWV nucleic acids.

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References:

Amin, P.W. and A.B. Mohammad. 1980. Groundnut pest research at ICRISAT. p. 158-166. In Proc. International Workshop on Groundnuts. 13-17 Oct, 1980. ICRISAT, Patancheru, India.

Clark, M.F. and A.N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of General Virology. 34: 475-483.

de Haan, L. Wagemakers, R. Goldbach and D. Peters 1989. Tomato spotted wilt virus, a new member of the Bunyaviridae? In Genetics and Pathogenecity of Negative Strand Viruses. Ed. By D. Kolakofsky and B.W. Mahy. pp. 287-290. Elsevier, Amsterdam.

Francki, R.I.B. and C.J. Grivell. 1970. An electron microscope study of the distribution of tomato spotted wilt virus in systemically infected Datura stramonium leaves. Virology 42: 969-978.

Ghanekar, A.M., D.V.R. Reddy, N. Iizuka, P.W. Amin and R.W. Gibbons. 1979. Bud necrosis of groundnut (Arachis hypogaea) in India caused by tomato spotted wilt virus. Annals of Applied Biology 93: 173-179.

Hobbs, H.A., D.V.R. Reddy, R. Rajeshwari, and A.S. Reddy. 1987. Use of direct antigen coating and protein A coating ELISA procedures for detection of three peanut viruses. Plant Disease 71: 747-749.

Ie, T.S. 1982. A sap-transmissible, defective form of tomato spotted wilt virus. Journal of General Virology 59: 387-391.

International Crops Research Institute for the Semi Arid Tropics (ICRISAT), 1978. Annual Report p. 153.

Laemmli, U.K. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T-4. Nature 277: 680-685.

Milne, R.G. and R.I.B. Francki. 1984. Should tomato spotted wilt virus be considered as a possible member of the family Bunyaviridae? Inter Virology 22: 72-76.

Mohamed, N.A., J.W. Randles, and R.I.B. Francki. 1973. Protein composition of tomato spotted wilt virus. Virology 56: 12-21.

Reddy, D.V.R. Groundnut Virus diseases: Distribution, Identification and Control. Review of Plant Pathology (In press).

Reddy, D.V.R., and J.A. Wightman. 1988. Tomato spotted wilt virus. Thrips transmission and control. In: Advances in Disease Vector Research. Ed. By K.F. Harris. Vol. 8. pp. 203-220. Springer Verlag - New York.

Spurr, A.R. 1969. A low-viscosity epoxy embedding medium for electron microscopy. Journal of Ultrastructural Research 26: 31-43.

Sudarshana, M.R. and D.V.R. Reddy. 1989. Penicillinase-based enzyme-linked immunosorbent assay for the detection of plant viruses. Journal of Virological Methods 26: 45-52.

Tas, P.W.L., M.L. Boerjan, and D. Peters. 1977. The structural proteins of tomato spotted wilt virus. Journal of General Virology 36: 267-279.

Towbin, H., T. Staehelin, and J. Gordon. 1979. Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. Proceedings of National Academy of Sciences, USA 76: 4350-4354.

Wongkaew, W., S. Sae-wien, D. Choopanya, M. Bhrammintra, W. Sakwong, and W. Sritongchai. 1985. Studies on virus diseases of groundnut. pp. 242. In Proc. of the 4th Thailand National Groundnut Research Meeting. 19-21 Feb. 1985. Khon Kaen University, Khon Kaen, Thailand.

Table 1. Host range of peanut yellow spot virus as determined by mechanical sap inoculations.

Host species	Symptoms[1]	
	Local	Systemic
Canavalia ensiformis	NL	-
Chenopodium amaranticolor	CL[2]	-[3]
C. quinoa	NL	-
Datura stramonium	CCL	-
Nicotiana benthamiana	CL	-
N. glutinosa	CL	-
Petunia hybrida	NL	-
Phaseolus vulgaris (cv. Topcrop)	CL[2]	-
Pisum sativum	NL	NL and VN[4]
Vigna radiata	NL	NL and VN[4]
V. unguiculata (cv C-152)	CL[2]	CCL[4]

[1] Virus infection was confirmed in ELISA tests CL chlorotic lesions; CCL chlorotic lesions with concentric rings; NL necrotic lesions; VN veinal necrosis.

[2] Older lesions had necrotic centers.

[3] Neither symptoms were observed nor virus was recovered in ELISA tests.

[4] Systemic infection observed only when ambient temperatures were between 20° to 30°C.

Table 2. Serological relationships of peanut yellow spot virus in PAC-ELISA.

Dilutions of peanut leaf extracts[1]		Antisera to PYSV	Antisera to TSWV (Indian isolate)	Antisera to TSWV[2] (Texas isolate)
Healthy	10^{-1}	0.09[3]	0.07	0.09
	10^{-2}	0.06	0.05	0.12
	10^{-3}	0.08	0.06	0.10
Infected with PYSV	10^{-1}	0.88	0.01	0.18
	10^{-2}	0.44	0.03	0.15
	10^{-3}	0.09	0.05	0.15
Infected with TSWV	10^{-1}	0.03	1.10	0.11
	10^{-2}	0.03	0.34	0.13
	10^{-3}	0.04	0.09	0.09

[1] Dilutions are based on original weight of tisuse.

[2] Supplied by Dr. J.W. Demski.

[3] Absorbance at 620 nm. Values are mean of absorbance values for three replicate wells, obtained after deducting from value for three buffer controls.

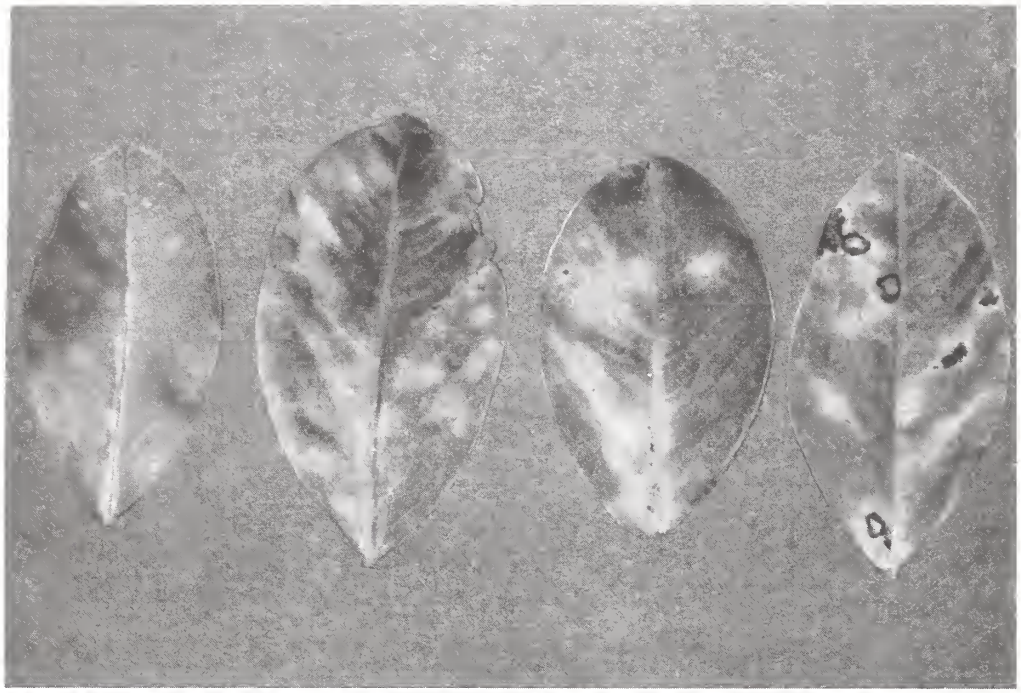

Fig. 1 Symptoms of peanut yellow spot virus on peanut leaflets. Symptoms development from initial (leaflet on left side) to advanced stage of disease development (leaflet on right side) is represented.

Fig. 2 Lesions produced on *Vigna unguiculata* cv. C 152.

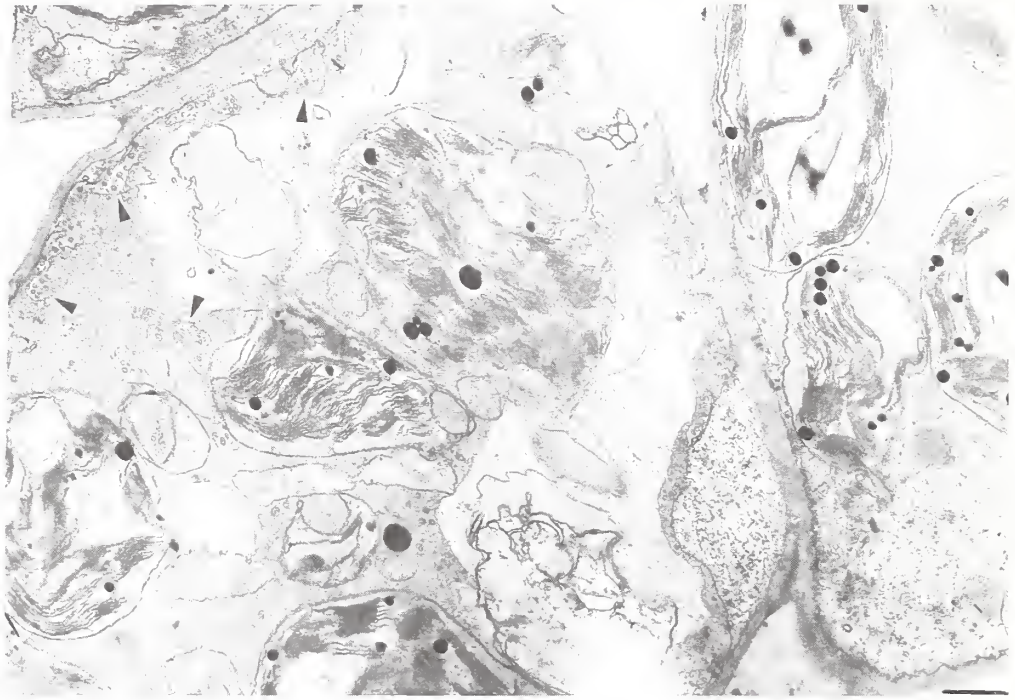

Fig. 3 Thin section of a peanut leaflet infected with peanut yellow spot virus. Bundles of virus particles enclosed in a membrane are shown by arrows Bar = 1000 nm.

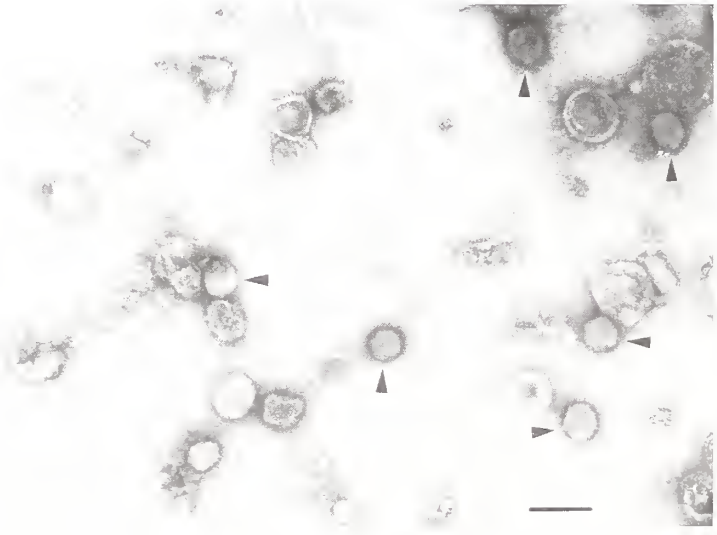

Fig. 4 Particles of peanut yellow spot virus. Bar = 140 nm.

TSWV: SYMPTOMS, HOST RANGE AND SPREAD

Ronald K. Jones and James R. Baker

Abstract

Symptoms of TSWV vary between plants, cultivars, species, time of year, plant age, etc. Chlorotic to necrotic lines, rings, blotches plus petiole, stem and veinal necrosis are common symptoms. Host range lists vary from 168 to 250 or more species of plants in at least 35 families and additional hosts are being reported constantly. The virus is being spread in the floral industry in vegetatively propagated plant material and infected thrips in plant shipments. The geographical range of the virus in the field is also expanding in North America.

Introduction

Tomato spotted wilt virus (TSWV) has become an increasingly severe problem for greenhouse floral growers over the past 5 years. The geographical range of the virus in the field has also been expanding in North America over the past 20 years.

The host range of TSWV include approximately 250 species of plants in at least 34 plant families (see Table 1). Plants most commonly infected in North Carolina include gloxinia, Rieger Begonia, exacum, New Guinea impatiens, garden impatiens, double flowered impatiens, tuberous dahlia, portulaca, cyclamen, cineraria and calceolaria. The disease has also been found in stephanotis, schefflera, aglaonema, zebra plant, strawberry geranium, wax begonia, 'Non Stop' begonia, spathiphyllum, anthurium, and others. A recent outbreak of TSWV was confirmed in hydrangea. This is particularly disturbing because hydrangea is becoming increasingly popular.

Spread of TSWV

The virus is spread by several species of thrips including the western flower thrips and by plant material moving in the industry. It is not spread in seed. During the first 3 months of 1990 we have detected six shipments of double flowered impatiens having a few infected plants. The virus can also be spread by infected thrips in plant shipments even on non-host plants.

Geographical Spread of TSWV in North America

TSWV was first found in greenhouse floral crops in Canada in the early 1980's and by the mid 1980's in the US. Now TSWV can probably be found in most greenhouses growing susceptible crops in the United States and Canada.

TSWV was observed in the field in Louisiana in the late 1960's and occurred in peanuts in east Texas in 1971. By the mid 1980's TSWV was reported in the field in California, across the Gulf coastal states to the western edge of Florida and north into Tennessee and Kentucky. In 1989, infected field grown tomato and pepper transplants were shipped from Georgia to many states in the northeastern and midwestern United States and Canada.

TSWV is now occurring in most southern states every year in peanuts, tomato, peppers, and tobacco. How far north the vectors and the virus can become established is a critical question.

Tomato Spotted Wilt Symptoms

Symptoms of TSWV vary between strains (mild to severe) of the virus, species of host plant, cultivars of a species and seasons. In gloxinia and New Guinea impatiens symptoms are more severe in the warm months. Symptoms

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Jones is a plant pathologist and Baker is an entomologist, North Carolina Agricultural Extension Service, North Carolina State University Raleigh, NC 27695

Table 1. Some hosts of Tomato spotted wilt virus.

Ornamentals

amaranthus
anaryllis
anemone
aster
baby's breath
bellflower
begonia
calceolaria
calendula
China aster
calla lily
chrysanthemum
cineraria
coreopsis
cosmos
dahlia
delphinium
evening primrose
forget-me-not
gaillardia
gerbera
gladiolus
gloxinia
hydrangea
lobelia
marigold
morning glory
nasturtium
peony
petunia
phlox
pineapple
poppy
primrose
ranunculus
salvia
snapdragon
sweet pea
tiger lily
verbena
zinnia

Vegetables

broad bean
cauliflower
celery
cow pea
eggplant
garden pea
lettuce
potato
pepper
snap bean
spinach
tomato

Field Crops

tobacco
peanuts

on cyclamen may be more severe in cool weather. Symptoms can appear on gloxinia within 5 days after inoculation in warm weather.

Gloxinia: Symptoms include necrotic line patterns and ring spots on leaves, terminal bud necrosis, malformed leaves, stunted plants, white ring and line patterns on colored flowers, flower distortion, and delayed flowering. The necrotic lines and ring patterns persist as long as the affected leaves live, but other symptoms may disappear. Symptoms expressions is most severe within 7-10 days after infection on young plants. If the plants survive this shock phase of the disease, they often become symptomless, of at least less severely damaged, but remain infected. Symptoms vary widely from plant to plant.

Hiemalis (Rieger) begonia: Symptoms include severe leaf vein necrosis, finely etched necrotic ring spots, chlorotic mottling and blotching. White rings (Color break) may occur in red and pink flower petals. Frequently, only 1 or 2 leaves show symptoms on individual plants. Plants may not appear to be severely damaged. Tomato spotted wilt has been observed in the cultivars 'Whisper O'Pink,' 'Renaissance,' 'Schwabenland Red,' and 'Improved Schwabenland Orange.'

Cyclamen: Finely etched necrotic and chlorotic ring spots occur in cyclamen leaves becoming brown necrotic spots. Plants may be stunted and flowers distorted. Damage to cyclamen can be minimal to severe.

Chrysanthemums: In several western states, chrysanthemums infected by TSWV exhibit severe necrosis on the stem and petioles. The cultivar 'Polaris' has been most severely damaged.

Over the past several years there have been increasing reports of TSWV on chrysanthemum in other parts of North America. Symptoms on chrysanthemum in North Carolina are defuse chlorotic blotches on leaves with slightly sunken tan lines or flecks within the blotches. Plant damage is very slight.

New Guinea Impatiens: On New Guinea impatiens symptoms include black ring spots and line patterns in leaves, black lesions on stems, slightly to extremely stunted plants, twisted and malformed leaves and terminal dieback. Symptoms vary widely from plant to plant and between cultivars. Cuttings from infected plants may fail to root or root and grow very slowly. Symptoms can be mild to severe. New Guinea impatiens in baskets hanging high in the greenhouse are very difficult to survey for symptoms.

Garden Impatiens: Symptoms include black ring spots and line patterns on leaves, black lesions on stems, leaf drop and stunted plants.

Exacum: Symptoms include tan leaf spots, necrotic terminals, wilted branches or sections of plants and brown cankers on the stems. The disease is more common in flowering size plants than young plants. Infected plants are usually unsaleable.

Cineraria: Symptoms include chlorotic spots on leaves, stunted plants and delayed flowering. Dark brown to black lesions develop on the petioles and leaf veins. Plants usually do not die but their quality is greatly reduced.

Calceolaria: Symptoms appear as water soaked, blighted wedge shaped parts of leaves.

Stephanotis: Symptoms include chlorotic ring spots in leaves and terminal dieback.

Diagnosis of Tomato Spotted Wilt Virus

Because symptoms of tomato spotted wilt vary with host plant species and even cultivar plus distribution of the virus particles in an infected plant is not necessarily uniform, diagnosis of TSWV is difficult. Some parts of the plant may have many particles and other parts may have few virus particles. Choosing the best tissue for assaying plants is critical. Efforts to refine detection techniques for TSWV are continuing. Immuno assay

techniques and indicator plants continue to be a major component of research work at North Carolina State University.

In the greenhouse floral industry there are 2 types or strains of the virus - the common strain and the impatiens strain. These two strains react completely different to antisera to each strain and there is no cross reaction between the two antisera. If a suscept plant is being tested serologically (by ELISA test) it must be tested against both antisera. Both antisera are available from Agdia Inc.

Control (Management of TSWV)

Control of TSWV has been difficult and/or expensive for many growers. It is very difficult to achieve the necessary level of control (100%) of the primary vector (western flower thrips) in commercial greenhouses growing numerous crop species on the floor, on the benches, with hanging baskets, with perennial plants on the wall (Stephanotis), with plants of all stages of growth from seedling to marketable sizes plus constantly bringing in new plant shipments possibly infested with thrips and/or infected with TSWV. Those growers who have had the most difficulty controlling TSWV trend to grow crops for the florist market (mums, Rieger begonia, large fancy gloxinia, exacum, etc.).

Exclusion of the Thrips Vectors

1. Purchase plants free of western flower thrips. This pesticide resistance thrips is an industry wide problem and it is frequently moved in plant shipments. It is difficult to impossible to inspect incoming plant shipments even for adults let alone the eggs and immature stages.

2. Screening greenhouse vents to exclude the thrips. Several major propagators are screening greenhouse vents with expensive stainless steel screens. VisQueen's original Vispore poly screen is being used by many growers but it is only partially effective because the holes are too large (400 holes per sq. in.) although Robb had spectacular results with screening in California. VisQueen is introducing a new material with smaller holes (1600 holes per sq. in.). All screening materials severely restrict air movement and therefore, can create cooling problems in warmer climates. To obtain adequate air movement for cooling in North Carolina we have suggested 4-5 times more surface area of the screened enclosure than the area of the cooling pads. Screening will become more important if TSWV continues to spread in the field. Remember, you can also screen the thrips in if they are introduced into a screened greenhouse on plant material or workers clothing.

Thrips Management in the Greenhouse

1. Survey: Monitor for thrips with yellow or blue sticky cards. Cards should be placed just over the crop and changed on a weekly basis. Thrips numbers and species should be recorded and tracked. Slightly higher thrips populations can be tolerated on non-host crops. Almost zero tolerance of western flower thrips should be maintained on susceptible crops. Frequency and type of insecticide can be programed based on the host and thrips populations. Even on susceptible crops, slightly higher thrips populations can be tolerated if the virus is not present, but this can be dangerous if the virus should be accidentally and unknowingly introduced.

2. Insecticides: Western flower thrips are highly resistant to most insecticides. Check with your local extension agent for legal recommendations. When spraying it is important to do it thoroughly. Spray all crops including hanging baskets and strive for complete coverage. Spraying only some crops may cause the thrips to move to other crops or areas in the greenhouse until the insecticide is no longer active. During serious outbreaks of western flower thrips in warm weather, it may be necessary to spray every 5 days or more.

3. Biological control: There is much current research work to develop biological control of western flower thrips and some progress is being made.

Resistant and Non-host Crops

Resistance occurs within host species where reaction to the virus varies from highly susceptible to very resistant. This apparently occurs in chrysanthemums with cultivars such as Polaris being highly susceptible and severely damaged by TSWV. Most pot mum cultivars grown in the east appear to be resistant and may only show chlorotic blotches in infected leaves and systemic virus movement may be restricted. Evaluation of resistance has not been reported on most ornamental species. Breeders have incorporated TSWV resistance into tomato but with limited success due to the highly variable nature (numerous strains) of the virus. There can also be resistance (lack of attractiveness) to the thrips vectors. This has been demonstrated in chrysanthemum by Allen and co-workers in Canada. Western flower thrips appear to be highly attracted to small vegetative gloxinia and they can express TSWV symptoms within 7-10 days after introduction into a greenhouse infested with TSWV carrying thrips. Exacum does not appear to be attractive to western flower thrips until flowering and the disease usually appears on market size plants. A non-host species is one that is immune to infection. Poinsettia is a non-host to TSWV and therefore, is not infected nor damaged by the virus even if virus carrying thrips feed on it.

An effective control measure then is to eliminate all susceptible host plants or cultivars such as gloxinia, exacum, Rieger begonia, Polaris chrysanthemum, etc. The occurrence of TSWV in many greenhouses is very low during winter months because poinsettia (non-host) is widely grown plus the thrips are less active during this time of year.

Eliminating Sources of the Virus

All infected plants must be promptly removed from the greenhouse at the first symptoms of the disease. This is only effective if done completely and regularly. One employee should be assigned to rogue plants on a regular schedule or all growers should be instructed to rogue daily. Erratic symptom expression and wide range of symptom types can make effective rouging very difficult. Perennial plants that only show symptoms occasionally can remain as unsuscepted sources of the virus. This effort is also unsuccessful if infected plants are being constantly reintroduced into the greenhouse. Even though infected plants such as Rieger begonia may be marketable, they still carry the virus and/or thrips and they should be dumped. Individual growers are at the mercy of the total industry effort to control this disease: Tomato spotted wilt can only be controlled by a total industry effort.

DIAGNOSTIC STRATEGY DEVELOPMENT FOR TOMATO SPOTTED WILT VIRUS

L. G. Brown[1], G. W. Simone[2], and R. G. Christie[3]

ABSTRACT

Tomato spotted wilt is of growing economic impact in Florida. In response to this, a diagnostic strategy is evolving using plant virus inclusions, serology and host inoculations. No single technique has been satisfactory in dealing with different strains and diverse hosts. The variable performance of these diagnostic methods is attributed to plant sample quality, sampling site, and variable virus titer over time. Therefore, a defined diagnostic strategy is needed for tomato spotted wilt virus.

Tomato spotted wilt virus (TSWV) became an economic concern in North Florida on tomatoes in 1986. Although this virus is now found all over North and Central Florida, the incidence within individual fields is usually below 10%. Isolated cases of TSWV have also been confirmed in South Florida tomato and pepper fields. Prior to 1990, only one greenhouse had a confirmed incidence of TSWV. Since then, four additional reports of TSWV in greenhouses with New Guinea impatiens, garden impatiens, gloxinia, and/or dahlias have been confirmed. Western flower thrips, Frankliniella occidentalis (Pergande), incidence in fields and greenhouses is also increasing in Central and South Florida (Division of Plant Industry insect records). This efficient vector of TSWV has been associated with outbreaks of the disease in field crops in North Florida and in greenhouse crops in North and Central Florida. With host, pathogen, vector, and favorable environmental conditions present for a TSWV epidemic, reliable diagnosis of TSWV becomes an even more important link to management of this disease.

The first method used to confirm TSWV in symptomatic plants in Florida was plant viral inclusions. Since this first diagnosis of TSWV in 1986, a diagnostic strategy utilizing viral inclusions, host inoculations and ELISA has evolved. This strategy can serve as a guideline for other diagnostic labs.

Understanding the environment in which a plant disease diagnostician works is important in the development of a useful diagnostic strategy. There are more than 90 government, private and commercially sponsored plant diagnostic laboratories in the U. S. Each laboratory has written or unwritten strategy for diagnosing diseases on the wide range of host material received. We (in Florida) feel like Grogan (7) that diagnosis and the maintenance of plant health comprises a complex and often frustrating problem. We try to gain systematized knowledge derived from observation, study, and experimentation. This is necessary to determine the nature and principles concerning the causation of disease. As part of this picture, an understanding of the factors affecting disease incidence and severity must be elucidated. Frustration develops because the diagnostician is obligated to the research methodology of plant pathology, but must do so with both speed and accuracy to provide a useful service.

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[1]Plant Pathologist, Florida Department of Agriculture and Consumer Services, DPI, Gainesville, Florida 32602, [2]Associate Professor, Plant Pathology Department, and [3]Biological Scientist IV, Agronomy Department, IFAS, University of Florida, Gainesville, Florida 32611.
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Diagnosticians deal with large numbers of samples from many crops and areas, therefore, acquiring both a grower specific and region or commodity-wide perspective on a pathogen. The diagnostician provides direct service on demand and is relied upon for a positive public image of agriculture to the taxpayers. They must also extend information back to researchers concerning the pathogen variation, incidence and performance in the field. Most diagnosticians face these increasing job responsibilities and increasing sample numbers with finite resources.

The diagnostic strategy to meet these responsibilities is a multistep sequence. The first step is the correct identification of the plant you are examining. A diagnosis on an improperly named plant can lead to improper pest management recommendations, misleading surveys, and lawsuits. With knowledge of the plant, indices of biotic and abiotic problems can be consulted to narrow the field of causal factors and direct attention to more specific literature. A crop history and patterns of disease distribution are useful in defining abiotic and biotic factors, but are sometimes difficult to obtain. A knowledge of the healthy crop appearance is essential in perceiving abnormal symptomatology. This includes methods of production, cultivar characteristics and the described diseases of the crop (7). Books, compendia, journals, and articles are the starting point but experience in the laboratory and field are invaluable. Grogan (7) and Streets (13) cover the details of these concepts.

Strategy development for a diagnostic laboratory dealing with TSWV is the model we plan to use as a basis of discussion. An integrated strategy for diagnosis of TSWV is built on experience (Fig. 1). A familiar host-pathogen combination that has been processed before in the laboratory is approached differently than an unfamiliar host pathogen combination. A direct approach, one that begins with virus testing, is applied to familiar host pathogen combinations. Other causal agents or abiotic factors are not dismissed but are not given top priority.

What are the factors involved with TSWV? Experience with the host is primary. When the commonly occurring problems for a host are understood then time is not spent searching for virus or virus-like pathogens when the cause is abiotic, fungal or bacterial. The symptoms of TSWV are well known to most of us for certain crops. The difficulty arises when an unfamiliar host is submitted. This is especially true for foliage and floral ornamentals. When the diagnostician has little experience with a host, a literature search is done to identify common diseases and disorders. Knowledge of general horticultural practices is essential.

A word of caution is necessary: some symptoms reported for TSWV are atypical of virus infection and are often attributed to other causes. Begonias illustrate the point. Typical TSWV symptoms on begonias vary from veinal necrosis to yellow or bronze ringspots, and blotches or mottling on the leaf blade. The bacterium, Xanthomonas campestris (Pamm.) Dows. pv. begoniae (Takimoto) Dye, produces a similar veinal necrosis and leaf spotting. Aphelenchoides fragariae (Ritzema-Bos), a foliar nematode, also causes a necrosis along the veins and other parts of the leaf usually beginning at the margin. All these symptoms are similar and therefore are confusing to a diagnostician with little experience with begonias. The obvious strategy is to check all the possibilities. The less obvious strategy is to use a combination of tests that will detect mixed infections, can be applied to many hosts and that are sensitive. No one test meets all these objectives but the correct combination of tests can.

The diagnostician needs both non-presumptive and presumptive methods of diagnosis. A method that directly visualizes the identifiable features of the pathogen is non-presumptive. The inclusion method and the electron microscope are examples. Presumptive methods such as c-DNA probes and serology, however, use markers that are visualized. The presumption is that the markers are joined to the virus in question. In a research environment the presumptive methods can become highly refined because one pathogen in one system is being explored. Here the etiology is known and the strategy is to work backwards in learning how to manipulate the pathogen. A diagnostician however works with very different pathogens and doesn't always have the resources to gain experience with a presumptive test for each pathogen.

The options available for the diagnosis of TSWV vary with the access to resource people associated with the diagnostic laborotary and the experience of the diagnostician. These options are outlined because methods common in research situations may not always be useful to the diagnostician under their constraints of time, space and economics. Three methods commonly available for diagnosis of TSWV are 1) serology, 2) cytological techniques for visualizing virus-induced inclusions, and 3) bioassay (11). Experience plays a key role in choosing which test to use. We have found that different hosts require different screening techniques. Gloxinia and impatiens have proven easy to diagnose with either ELISA, inclusion, or inoculation. Whereas tomato and begonia sometimes require all three. This may be due more to the uneven distribution of the virus (8) and/or to sample quality which often can deteriorate quickly after collection.

ELISA is being used in many diagnostic laboratories because antisera for some TSWV isolates are available both commercially and privately. ELISA is efficient for a large number of samples and is a sensitive test. This sensitivity allows multiple site sampling which is important with viruses like TSWV which appears to be unevenly distributed in the plant (8). These benefits aside, ELISA is a presumptive test. The tester is presuming that the system is not giving a false positive or false negative. Infection by more than one virus is also not detected by ELISA. This is something the diagnostician must always consider.

Plant viral inclusions have been shown in many hosts to be objective intracellular evidence of viral infection (4,5,10). Inclusions induced by viruses of a number of virus groups maintain a characteristic appearance over a host range. Many of these inclusions can be detected with a compound light microscope when they are properly stained (4). The light microscopic approach to virus detection is based on the use of dyes capable of staining the cytological structures (inclusions) that result from viral infection. Samples can be stained and mounted in 20 to 40 minutes. However, the slides require an additional 5-10 minutes to survey. The major difficulties with this technique lie in learning to recognize the inclusion types induced by different virus groups and to sample tissue areas properly. However, this skill is readily acquired through practice and experience.

The most conspicuous inclusions induced by TSWV, as determined by cytological studies with the electron microscope, consist of virus aggregates contained within a convoluted system of membranes. These structures stain with both protein and nucleic acid stains. Their unique appearance and staining properties make it possible to distinguish these structures from 1) host constituents, 2) anomalies induced by non-viral plant pathogens, and 3) inclusions induced by other virus groups. The ability to differentiate TSWV inclusions from those induced by viruses in other groups makes this microscopic technique particularly valuable for the determination of multiple viral infections, a problem commonly encountered in field samples.

Inclusions are the first screen for viral pathogens that is used in our laboratory. The technique is most helpful when the symptoms are poor but no other pathogens are detected. When detected they will provide visualized (non-presumptive) evidence of the virus. Inclusions can be used as a screen to pick-up TSWV from hosts with atypical symptoms or to get some indication that TSWV is not a factor. The diagnostician must realize that the absence of inclusions, like a negative result from ELISA and host inoculations, is not absolute proof that TSWV or some other agent is not present.

The inclusion technique is the only rapid and inexpensive, non-presumptive technique for viral diseases that can detect multiple infections in the same plant. DPI's laboratory received dahlia samples that had classic TSWV symptoms which included ringspots and chevron patterns on the leaves. Some plants had both yellowish or pale green vein banding and ringspots while others had one symptom type only. Vein banding is symptomatic of dahlia mosaic virus (DaMV) (1,9,12). Cytoplasmic inclusion bodies found in plants infected with DaMV are structurally similar to those induced by other members of the caulimovirus group (1,9,12). Cytoplasmic inclusions representative of TSWV and DaMV (9) were detected in the same plant from tissue with symptoms characteristic of both viruses. Both the inclusion and ELISA techniques were used to make the diagnosis of TSWV. Host inoculation tests for DaMV were not used initially because they required production of non-traditional inoculation hosts and use of non-traditional methods of grinding host material in liquid nitrogen. These methods are required because dahlia contains inhibitory substances that greatly reduce transmission of the virus from leaf extracts (9). Although antisera to DaMV is available, most laboratories will not stock this item unless they routinely receive many dahlia samples. Private testing services , however, are available to process dahia mosaic samples.

Healthy controls are always helpful with the inclusion body technique but are not always available. With ELISA, a presumptive test, a positive reaction, without negative controls, is also difficult to interpret. The questions of host reactivity and proper testing conditions must be considered. Although inclusions are directly visualized and therefore may lend more confidence when diagnosing TSWV with a host for the first time, they are not infallible. Correlation of inclusions with physiological age of the infected plant, time since infection, and plant anatomy is necessary. These factors may influence the detection of viral inclusions as well as ELISA and host indicator tests.

Sap inoculation using sensitive indicator hosts such as Nicotiana glutinosa L. and Chenopodium quinoa Willd. provides much useful information. Indicator host ranges can separate mixed infections and differentiate local isolates (3,14) based on symptomatology. Though useful, such reliance on indicator hosts is usually beyond the budget, staffing, greenhouse capacities, and time requirements of most diagnostic laboratories. Therefore, many facilities use host indicators to established infectivity in hosts that are new to the laboratory and other methods are used to diagnose the pathogen.

Each laboratory has preferred inoculation hosts and a buffer system that gives results for TSWV. We use a 0.05M potassium phosphate buffer at pH 7.2 with 0.5% 2-mercaptoethanol added. This buffer system has been consistent in performance. A critical test of the various formulas would likely demonstrate that TSWV can be successfully inoculated using many different buffers. The common denominator with buffers seems to be a pH of 7.0-7.2, a molality of 0.05 and mercaptoethanol or sodium sulfite used as additives (1,2,6,11).

With suspected new hosts of TSWV all three techniques are needed to confirm the diagnosis. With growing diagnostic experience on a new host for TSWV, one or two techniques may be found adequate for viral diagnosis. Inclusions are a good, non-presumptive first screen, providing there is reasonable tissue quality. ELISA has also been a good choice to screen for TSWV but the process is not efficient with a small number of samples. Undefined TSWV strains (15) and the problem of sample quality also hamper the usefulness of ELISA.

There are several isolates of TSWV and symptomatology can vary among isolates and within and among different hosts. The issue of isolates and strain differences always has to be considered by the diagnostician. These questions are complex and are addressed in other workshop presentations. The diagnostician should consider how different strains or isolates may affect the diagnosis. However, with TSWV the issues are still being addressed by the researchers. The definitive work that will aid the diagnostician is still ongoing. The conscientious diagnostician will attempt to stay informed about the dynamic TSWV strain picture.

Documentations of TSWV diagnoses (with diagnostic methods) should be made on local, state and national networks. The resulting data bases and and reports confirming diagnostic techniques save diagnosticians time in dealing with this pathogen. TSWV presents an opportunity for research, extension, regulatory, and private diagnostic laboratories to cooperate in formal and informal ways to resolve the difficult problem of diagnosis of TSWV. Our research in Florida will correlate the utility of ELISA, indicator hosts and plant viral inclusions with time course studies to improve sampling for diagnosis.

REFERENCES.

Brunt, A. A. 1971. Dahlia mosaic virus. CMI/AAB Descriptions of Plant Viruses No. 51. Surrey, England. 4 pp.

Black, L. M., Brakke, M. K., and Vatter, A. E. 1963. Purification and electron microscopy of tomato spotted wilt virus. Virology 20:120-130.

Cho, J. J., and Reddyman, H. B. 1988. Identification and separation of TSWV biological strains. Phytopath. 78:1548 (abst.).

Christie, R. G., and Edwardson, J. R. 1986. Light microscope techniques for detection of plant virus inclusions. Plant Dis. 71:273-279.

Christie, R. G., and Edwardson, J. R. 1977. Light and electron microscopy of plant virus inclusions. Fla. Agric. Exp. Sta.Monogr. 9. 155 p.

Francki, R. I. B., and Hatta, T. 1981. Tomato spotted wilt virus. Pages 492-511. in E. Kurstak (ed.). Handbook of Virus Infections and Comparative Diagnosis. Elsevier/North Holland Biomedical Press.

Grogan, R. G. 1981. The science and art of plant-disease diagnosis. Ann. Rev. Phytopathol. 19:333-51.

Holmes, F. O. 1955. Elimination of spotted wilt from Dahlias by propagation of tip cuttings. Phytopath. 45:224-226.

Lawson, R. H., and Taconis, P. J. 1965. Transfer of DaMV with liquid nitrogen and relation of transfer to symptoms and inclusions. Phytopath. 55:715-718.

McWhorter, F. P. 1965. Plant viral inclusions. Ann. Rev. Phytopathol. 3:287-312.

Nameth, S., and Barnes, L. 1990. A plant disease diagnostic sheet. Diagnostician's Quarterly. 11(3):in press.

Shephard, R. J. and Lawson, R. H. 1981. Caulimoviruses. Pages 847-878. in E. Kurstack (ed). Handbook of Virus Infections and Comparative Diagnosis. Elsevier North Holland Biomedical Press.

Streets, R. B. 1979. The Diagnosis of Plant Diseases. University of Arizona Press.

Tsakiridis. J. P., and Gooding, G. V. Jr. 1972. Tomato spotted wilt virus in Greece. Phytopath. medit. 11:42-47.

Wang, M., and Gonsalves, D. 1990. ELISA detection of various tomato spotted wilt virus isolates using specific antisera to structural proteins of the virus. Plant Dis. 74:154-158.

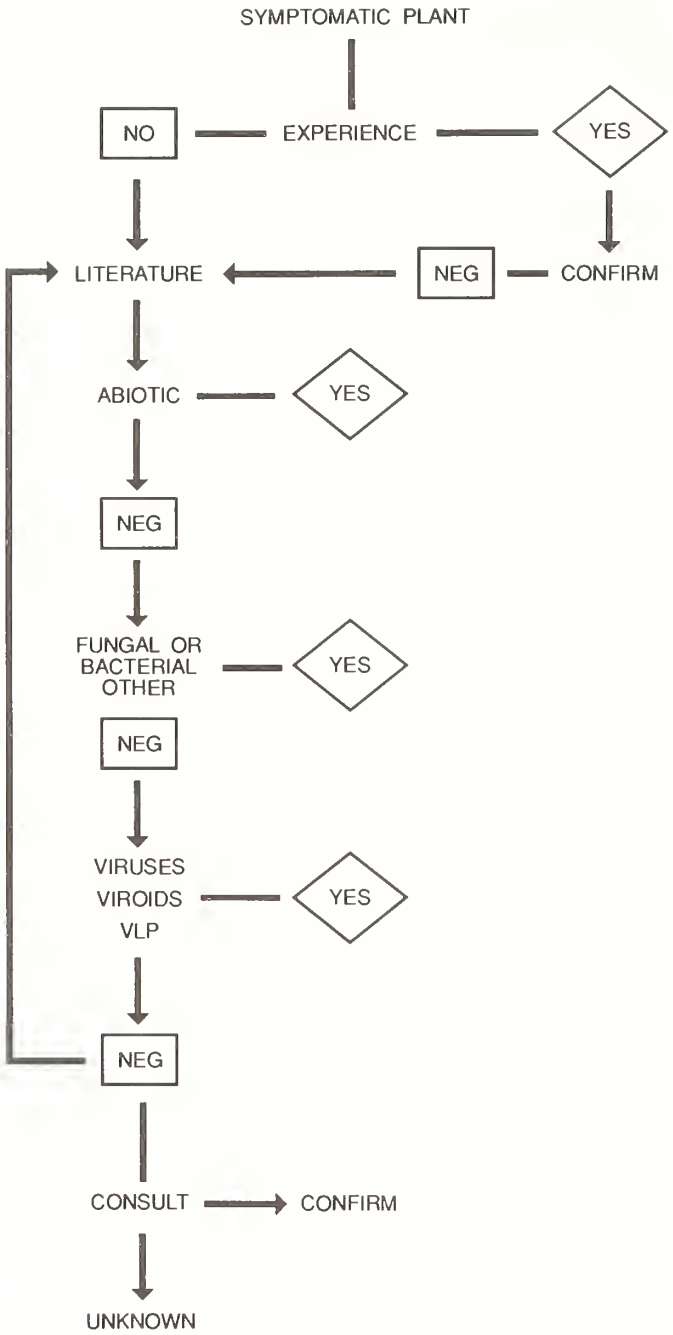

FIG. 1. DIAGNOSTIC STRATEGY FOR TSWV

TWO METHODS OF PRODUCING MOUSE MONOCLONAL ANTIBODIES FOR TOMATO SPOTTED WILT VIRUS

John L. Sherwood and Hei-ti Hsu

ABSTRACT

Two different approaches were utilized to induce immune responses in BALB/c mice for production of hybridomas secreting monoclonal antibodies to tomato spotted wilt virus (TSWV). In a conventional immunization procedure, adult mice were intramuscularly injected four times with a purified TSWV preparation over a period of three weeks (Phytopathology 79:61-74). Alternatively, when purified TSWV antigens were difficult to attain, a partially purified virus immunogen preparation was injected into adult mice which induced immunological tolerance to normal host plant antigens (tolerogens) (Phytopathology 80:158-162). Suppression of immune response to normal host plant antigens was induced by injecting neonatal mice four times with tolerogens. Hybridomas were produced using spleen cells derived from mice injected with partially purified TSWV preparations. Both approaches lead to successful production of monoclonal antibodies to TSWV.

INTRODUCTION

Tomato spotted wilt virus (TSWV) is the type member of the tomato spotted wilt virus group, and there are a large number of strains of the virus (Francki and Hatta 1981). The virus is vectored by thrips and infects a wide range of plant species in many families (Best 1968; Cho et al., 1986; Francki and Hatta 1981). There is a critical need for reliable diagnostics for TSWV, because TSWV-infected plants may not show severe symptoms until the onset of flowering or following a period of stress.

TSWV is enveloped with an isometric morphology and a diameter of about 85 nm. Three virus specific glycoproteins of 52 kDa, 58 kDa, and 78 kDa are present in the virus envelope, and the nucleocapsid has a protein of 29 kDa (Mohamed et al., 1973). Although many procedures for purifying TSWV have been reported (Black et al., 1963; Francki and Hatta 1981; Gonsalves and Trujillo 1986; Mohamed et al., 1973), it can be difficult to prepare sufficient amounts of purified virus suitable for immunization. Purified TSWV is unstable and rapidly breaks down once outside the tissue of its hosts (Black et al., 1963).

TSWV antigen may be detected serologically in diseased plants (Cho et al. 1986; Gonsalves and Trujillo 1986), but disease diagnosis is restricted by the lack of availability of high quality antisera (Francki and Hatta 1981). Suitable immunogens for TSWV (Alden 1956) were first prepared more than 30 yr ago by using a mild extraction method (Black and Brakke 1954) and a density gradient centrifugation procedure (Brakke 1951). Although the titers of the antisera were low, they reacted specifically to intact TSWV and soluble antigens in subviral preparations in ring precipitin tests. Only recently, was an improved antiserum prepared for a strain of TSWV (Gonsalves and Trujillo 1986) by purifying the virus using a procedure described by Black et al. (1963) and modified by Mohamed et al. (1973).

Sherwood is an Associate Professor, Oklahoma State University, Stillwater, OK 74078; Hsu is a Microbiologist, USDA-ARS, Florist and Nursery Crops Laboratory, Beltsville, MD 20705. Journal article no. J-5678 of the Oklahoma Agricultural Experiment Station, Oklahoma State University, Stillwater.

Techniques for hybridization of myeloma cells with spleen cells secreting antibody permit the production of unlimited amounts of antibodies from immortal hybridoma cell lines (Kohler and Milstein 1975). The use of hybridoma technology to produce antibodies for the detection of enveloped plant viruses has been lacking. When an antigen cannot be extensively purified, it can be difficult to produce and select hybridomas producing antibody to the target antigen because of the high proportion of non-target antibodies and non-target antigens present. Enhancement of the proportion of spleen cells secreting antibodies to target antigens prior to cell hybridization is possible. A method for enrichment of specific antibody-producing splenocytes prior to cell fusion has been reported for cauliflower mosaic virus (George and Converse 1988). Another approach to achieve the enhancement of specific antibody-producing hybridomas is by induction of immunological tolerance to non-target antigens. This can be achieved by injecting excess amounts of an antigen into neonatal animals, and is characterized by the absence of detectable antibodies to that antigen (Felton and Ottinger 1942).

The purpose of this paper is to present results of two approaches for the development of monoclonal antibodies (MAB) to TSWV. One approach focuses on the process of selecting of hybridomas secreting MAB to TSWV when a purified preparation of TSWV was used as an antigen. The other approach focuses on a method of enhancing the proportion of TSWV-specific antibody secreting hybridomas by suppressing development of non-target antibodies to host plant constituents in mice. Related reports have been published (Hsu et al., 1990; Sherwood et al., 1989)

MATERIALS AND METHODS

The research presented in this paper was done independently in the laboratories of the two authors (Hsu et al., 1990; Sherwood et al. 1989). Therefore, the materials and methods, and results sections are divided into two distinct sections for clarity of presentation.

Use of Purified TSWV as an Antigen

Viruses and purification

The TSWV isolate (TSWV-OK) used for MAB production was maintained in Datura stramonium L. and purified according to the method of Mohamed, et al (1973). Other isolates of TSWV were obtained from H. Scott, University of Arkansas (TSWV-AR); B. Reddick, University of Tennessee (TSWV-TN); J. Cho, University of Hawaii (TSWV-T1 and TSWV-T2), L. Lane, University of Nebraska (TSWV-NE); and J. Moyer, University of North Carolina (TSWV-Type and TSWV-I). These isolates were also maintained in D. stramonium, except TSWV-I which was maintained in Nicotiana benthamiana Domin. In addition, the TSWV-T1 and TSWV-T2 isolates were maintained in Lycopersicon esculentum Mill., and the TSWV-TN isolate was maintained in Nicotiana tabacum L. These are the hosts from which these isolates were originally obtained. Other viruses used in serological assays were maintained in appropriate hosts. Polyclonal rabbit antiserum to TSWV was obtained from D. Gonsalves (Gonsalves and Trujillo, 1986).

Hybridoma production, screening and isotyping

Hybridomas were produced using methods previously reported (Sherwood et al., 1989). Briefly, Balb/c mice were immunized by intramuscular injection with TSWV obtained from 100 g of infected D. stramonium on four separate times over a three week period. The first three injections were made 1 wk apart and the virus was emulsified in Freund's complete adjuvant. The final injection was made 3 days before fusion and the virus was mixed in distilled water. Spleens were aseptically removed and spleen cells were

fused with the mouse myeloma cell line P3X63Ag8.653 using PEG-1000. After fusion, hybridomas were selected in HAT medium.

Hybridomas were screened for antibody production by a modified protein-A sandwich enzyme linked immunosorbent assay (PAS-ELISA) (Edwards and Cooper 1985). Plates were first coated with 1 µg/ml protein-A (Sigma Chemical Co. No P6650, St. Louis, MO) in 0.05 M carbonate buffer, pH 9.6, for 2 hr at room temperature. Plates were rinsed 3 times with phosphate buffered saline containing 0.05% Tween (PBS-Tween), and then a 1/2000 dilution of anti-TSWV polyclonal antiserum in PBS-Tween was added. After incubation at room temperature for 2 hr, plates were rinsed 3 times with PBS-Tween and either TSWV infected D. stramonium or uninfected D. stramonium, macerated and diluted 1/100 (w/v) in PBS-Tween with 2% polyvinyl pyrrolidone (PVP), was added. After overnight incubation at 4 C, plates were rinsed 3 times with PBS-Tween and undiluted culture supernatant was added to plate wells. After a 2.5 hr incubation, the plates were rinsed 3 times with PBS-Tween and alkaline phosphatase linked goat anti-mouse IgG at the manufacturer's recommended working dilution was added (Sigma Chemical Co. No A5153). After an additional 2.5 hr, the plates were rinsed as above and p-nitrophenyl phosphate in diethanolamine substrate buffer (1 mg/ml) was added.

Hybridoma cell lines which gave positive reactions only to the TSWV infected material were cloned using a soft agar method as previously reported (Sherwood et al., 1987). The subclass of immunoglobulin produced by each hybridoma line was determined with a mouse isotype identification kit (Zymed Laboratories, So. San Francisco, CA).

MAB was isolated by 50% saturated ammonium sulfate precipitation of culture supernatant of hybridomas grown in HL-1 serum free medium (Ventrex, Portland, ME). The precipitate was dialyzed against three changes of PBS prior to use.

ELISA and dot-immunobinding assays

To determine the serological reactivity of the MAB to different plant viruses, the modified PAS-ELISA was used as outlined above except that the polyclonal capture antibody was homologous to the virus being tested. Except for the TSWV capture antibody, which was used at a 1:2000 dilution, the capture antibodies were used at a 1:1000 dilution. Direct double-antibody sandwich ELISA (DAS-ELISA) was conducted according to Clark and Adams (1977). Plates were coated for 2 hr with 10 µg/ml MAB in coating buffer, and then rinsed with PBS-Tween. Samples diluted in PBS-Tween containing 2% PVP were added to the wells. After overnight incubation at 4 C, plates were rinsed 3 times with PBS-Tween, and a 1:400 dilution of alkaline phosphatase (Sigma Chemical Co. No P5521) conjugated to anti-TSWV MAB was added. The MAB was conjugated to alkaline phosphatase using the same procedure outlined by Clark and Adams (1977) for the conjugation of polyclonal IgG to the enzyme. After incubation at room temperature for 4-6 hr, plates were rinsed with PBS-Tween and the substrate added. Plates were incubated and then analyzed with a BIO-TEK EIA plate reader (BIO-TEK Instruments, Inc., Burlington, VT).

PAS-ELISA was carried out as above except that MAB was used as both the capture and probe antibody at 100 µg/ml, and protein-A alkaline phosphatase (Sigma Chemical Co. No P9650) at 1 µg/ml in PBS-Tween was used to detect the binding of the probe antibody to the sample.

For the dot-immunobinding assay (DIBA), tissue samples were prepared by grinding in Tris-buffered saline (TBS) and centrifuged at 6,000 g for 10 min. Samples prepared in this manner did not differ in infectivity compared to samples not centrifuged when inoculated to Petunia hybrida Vilm., a local-lesion host for TSWV.

Samples of 4 µl were spotted on nitrocellulose (NC) sheets and allowed to air dry. Samples on NC sheets were soaked in a 5% solution of Carnation non-fat dry milk for 30 min. NC sheets were then incubated in a 10 µg/ml solution of MAB in TBS for 1 hr. After rinsing in TBS, the NC sheets were incubated for 1 hr in goat anti-mouse alkaline phosphatase (1:1000 dilution in TBS). The NC sheets were rinsed in TBS and then incubated in a substrate of 5 mg nitroblue tetrazolium in 15 ml of 0.1 M Tris buffer, pH 9.5, with 0.1 M NaCl and 5 mM $MgCl_2$ with 2.5 mg of 5-bromo-4-chloro-3-indolyl-phosphate in 50 µl of N,N-diethyl-formamide (Leary et al., 1983).

To determine the optimal concentration of reagents in both the DAS-ELISA and the PAS-ELISA, the capture and probe antibodies or probe conjugate were added at various concentrations. For the DIBA assay, different incubation times and different dilutions of MAB were used to determine satisfactory conditions for the assay.

Identification of proteins reacting to MAB to TSWV

To determine to which of the proteins of TSWV the MAB produced reacted, the virus was dissociated and the proteins separated according to the method of Verkleij and Peters (1983). TSWV was dissociated by the addition of Nonidet-P40 (NP40) to a 1 ml suspension of virus obtained from 50 g of D. stramonium to a final concentration of 2%. The viral glycoproteins were separated from the nucleocapsid protein by centrifugation through a sucrose gradient and the proteins concentrated by dialysis (Verkleij and Peters 1983). The reaction of the MAB to the protein fractions was tested in ELISA and the protein composition of each fraction analyzed by SDS-polyacrylamide gel electrophoresis (Laemmli 1970).

Induction of Immunotolerance to Non-target Antigens

Viruses

Isolates of TSWV were maintained in Datura stramonium L. at Geneva, NY. An isolate from Oklahoma (TSWV-OK) from John L. Sherwood of Oklahoma State University and an isolate from Hawaii (TSWV-BL from Batavia lettuce, Lactuca sativa L.) (Gonsalves and Trujillo 1986) were used in the present study. Viral antigens were prepared at Cornell University, Geneva, NY, according to a procedure previously described (Gonsalves and Trujillo 1986).

Preparation of plant antigens (tolerogens)

Healthy D. stramonium antigens were prepared by the same procedure used for viral antigens (Gonsalves and Trujillo 1986). After sucrose density gradient centrifugation in a Beckman SW28 rotor, materials located between 3-6 cm from the bottom of tubes were collected and pelleted by centrifugation at 300,000 g for 60 min in a Beckman Type 50.2 Ti rotor at 4 C. The resulting pellets were resuspended in phosphate-buffered saline (PBS, 0.02 M phosphate, 0.15 M NaCl, pH 7.4) to give a 50-fold concentration of plant antigens (i.e., 50 g of tissues resulted in 1 ml of plant antigen preparation in PBS). The resuspended pellet was stored in 100-µl aliquots at -70 C until use.

Mice

Pregnant and 20-g female virgin adult BALB/c mice were obtained from Dominion Laboratories (Dublin, VA). Pregnant mice were observed daily for newborns. Gloves were worn for handling newborns and 26 G or 27 G needles were used for injecting newborns.

Injections

Six newborn mice from the same litter were each injected intraperitoneally with 25, 40, 100, and 200 µl of healthy

D. stramonium antigens (tolerogens) on days 1, 3, 5, and 7, respectively. Six sibling mice from a second litter did not receive healthy plant antigens (tolerogens) injections and were used as controls. Two mice at 5 wk, 2 at 7, and 2 at 9 wk for each group were immunized intraperitoneally with a single injection of 200 µl of a TSWV preparation when the mice were 5, 7, or 9 wk old. Splenectomies were performed 4 days after the immunization, and fusions were made immediately.

For immunization of adult mice, a conventional injection schedule was employed as previously reported (Hsu et al., 1984). Six 20 g-female mice were injected 3 to 4 times with 200 µl each of TSWV preparations per injection. Mice in all treatment groups were sacrificed 4 days after the final injection, and spleen cells were prepared for hybridization.

Myelomas, fusions, and culture media

The myeloma cell line P3/NS1/1-Ag4-1 used in previous studies (Hsu et al., 1984) was used for fusions. The preparation of myeloma cells, single splenocyte suspensions, and methods of fusion were similar to those previously described (Hsu et al., 1984).

ELISA

Two types of indirect ELISA were used to identify hybridoma cultures secreting antibodies specific to TSWV or normal host antigens. Before adding hybridoma culture supernatants, TSWV antigens were trapped on polyvinyl chloride (PVC) plates either by: A) direct coating at pH 9.6 in 0.1 M carbonate buffer (Hsu and Lawson 1985) or B) incubating a TSWV preparation in PVC plates coated with rabbit anti-TSWV serum at pH 7.4 in PBS at room temperature for 2 hr (Hsu et al., 1984). Rabbit anti-TSWV sera were prepared at Cornell University (Gonsalves an Trujillo 1986). The use of either one of the prepared ELISA plates alone may affect the selection of antibodies suitable only in one type of ELISA but not the other (Hsu et al., 1988). For controls, concentrated extracts from healthy D. stramonium were used instead of the virus antigens. Hybridoma culture fluids from each well were collected at two different times and were tested separately.

RESULTS

Use of Purified TSWV as an Antigen

MAB production and characterization

Of the initial 62 hybridomas that tested positive by ELISA for production of antibodies to TSWV, one had strong reactivity to TSWV in ELISA and did not appear to react with any of the viruses tested. The viruses tested included five potyviruses, wheat soilborne mosaic virus, tobacco mosaic virus, cucumber mosaic virus, tobacco streak virus, and brome mosaic virus. Because of the superior performance of MAB from clone 1-7B4, it was used for the remainder of the research. This clone produced immunoglobulin of the IgG2b subclass.

Table 1. Reaction of tomato spotted wilt virus (TSWV) specific monoclonal antibody 1-7B4 in double antibody sandwich (DAS) ELISA, protein-A sandwich (PAS) ELISA, and a dot-immunobinding assay (DIBA) to isolates of TSWV in different hosts.[a]

	DAS-ELISA			PAS-ELISA			DIBA
		Absorbance (A405nm)			Absorbance (A405nm)		
Virus isolate and host	Dilution of virus sample	Virus sample	Healthy control[b]	Dilution of virus sample	Virus Sample	Healthy control	Maximum dilution detected[c]
TSWV-OK	1/1280	0.734±0.012	0.006±0.002	1/1280	0.332±0.015	0.008±0.004	1/1280
D. stramonium	1/2560	0.198±0.011	do	1/2560	0.248±0.023	do	
	1/5120	0.039±0.007	do	1/5120	0.051±0.007	do	
TSWV-TN	1/1280	0.543±0.021	0.002±0.001	1/1280	0.335±0.033	0.012±0.004	1/640
tobacco	1/2560	0.157±0.011	do	1/2560	0.228±0.056	do	
	1/5120	0.007±0.002	do	1/5120	0.036±0.009	do	
TSWV-T_1	1/1280	0.057±0.011	0.001±0.001	1/1280	0.063±0.011	0.003±0.001	1/320
tomato	1/2560	0.019±0.008	do	1/2560	0.018±0.008	do	
	1/5120	0.007±0.002	do	1/5120	0.004±0.001	do	
TSWV-T_2	1/1280	0.023±0.010	0.003±0.001	1/1280	0.019±0.003	0.005±0.002	1/320
tomato	1/2560	0.015±0.005	do	1/2560	0.012±0.003	do	
	1/5120	0.003±0.002	do	1/5120	0.004±0.001	do	

[a] See text for concentration of serological reagents used. Plates read after a 30 min incubation.
[b] Healthy control material was diluted 1/10 in ELISA tests.
[c] Maximum dilution detected determined by intensity of sample spot compared to control at an equal dilution.

Table 2. Reaction of TSWV-specific MAB 1-7B4 to Nonidet-P40-disrupted TSWV in double-antibody sandwich ELISA (A_{405}nm).[a]

Virus proteins present[b]	ELISA value
52 kDa, 58 kDa, 78 kDa	0.004
29 kDa	0.435
Nondisrupted Control	0.575
Phosphate buffered saline	0.002

[a] Plates were coated with 10 µg/ml anti-TSWV. Samples were probed with a 1:400 dilution of alkaline phosphatase conjugated to anti-TSWV MAB.

[b] Samples of proteins were obtained from TSWV obtained from 50 g of D. stramonium. Nondisrupted control was a 1:1000 dilution of virus obtained from 50 g of D. stramonium.

Induction of Immunotolerance to Non-target Antigens

In mice injected first with normal host antigens (tolerogens) followed by the viral antigens, percentages of TSWV-specific hybridomas were considerably greater (Table 3) than in those mice of the same age that received only the viral antigen (Table 4). Although the lowest number of virus-specific hybridomas was derived from mice immunized first with healthy plant antigens when they were 5 wk old, the percentage of virus-specific hybridomas was higher in these samples than it was among animals 7 or 9 wk old when injected with viral antigen (Table 3). The average percentages of TSWV-specific hybridomas derived from 5-, 7-, and 9-wk-old tolerance-induced mice were 83, 50, and 40% respectively. The percentages of TSWV-specific hybridomas derived from tolerance-induced mice were comparable for both strains of TSWV when tested at 5 and 9 wk (Table 3). We were not able to collect data for the TSWV-OK strain for the 7-wk-old mouse injected with healthy plant antigens because of spleen cell loss.

Fewer TSWV-specific hybridomas were generated from control mice that were not preimmunized with healthy plant antigens. No TSWV-specific hybridomas were produced from 5-wk-old mice that were not immunized first with plant antigens (Table 4). On the other hand, numbers of hybridoma antibodies reactive to plant antigens were fivefold greater than those obtained for mice of the same age first injected with healthy plant antigens (Tables 3 and 4). Between 5 and 10% of the hybridomas produced in mice immunized at 7 or 9 wk with TSWV preparations were virus specific (Table 4). The number of hybridomas secreting antibodies specific to TSWV-OK, TSWV-BL, or non-target host plant antigens was greater from mice immunized at 9 wk than from mice immunized at 5 wk (Tables 3 and 4).

Hybridomas secreting TSWV-specific antibodies varied from 3 to 9 per spleen/fusion for those mice that only received multiple immunizations of TSWV (Table 5). Multiple immunizations produced a larger number of plant antigen-specific hybridomas ranging from 65 per spleen/fusion to as many as 185 per spleen/fusion (Table 5) than for a single immunization (Table 2 and 4).

DISCUSSION

Francki and Hatta (1981) indicated the serological diagnosis of TSWV has not been adequately addressed. Although polyclonal antiserum of excellent quality can be produced to this virus (Gonsalves and Trujillo 1986), there are few reports of this being accomplished. The MAB technology has been shown to have the potential to address the problem of producing significant

Table 3. Specificities (number and percentage) of hybridomas derived from tomato spotted wilt virus (TSWV) immune splenocytes of mice neonatally injected with plant constituents followed by injection with strain BL or strain OK at the age of 5, 7, or 9 wk[a]

Age of mice when immunized with TSWV	Hybridoma specificities								Percentage of virus specific hybridomas
	BL-immunization				OK-immunization				
	Virus specific		Host specific		Virus specific		Host specific		
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	
5	6	86	1	14	9	82	2	18	83
7	11	50	11	50	...[b]	...	...[b]	...	50
9	30	41	44	59	23	39	36	61	40

[a] Newborn mice were injected intraperitoneally with 25, 40, 100, and 200 µl of partially purified healthy Datura stramonium antigens, respectively, on day 1, 3, 5, or 7. A single immunization of 200 µl of TSWV-BL or TSWV-OK preparation was followed when mice were 5, 7, and 9 wk old. Splenectomies were performed 4 days after immunization for each age group.

[b] Loss of cells occurred during the procedure.

Table 4. Specificities (number and percentage) of hybridomas from pairs of mice, each pair receiving a single immunization of tomato spotted wilt virus (TSWV-BL or TSWV-OK) when 5, 7, or 9 wk old[a]

Age of mice when immunized with TSWV	Hybridoma specificities								Percentage of virus specific hybridomas
	BL-immunization				OK-immunization				
	Virus specific		Host specific		Virus specific		Host specific		
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage	
5	0	0	9	100	0	0	7	100	0
7	1	6	17	94	2	9	21	91	7
9	5	8	54	92	4	6	59	94	7

[a] Six sibling mice from the same litter were immunized with 200 µl of TSWV-BL or TSWV-OK preparation at the age of 5, 7, or 9 wk. Splenectomies were performed 4 days after immunization for each age group. Note, these are standard controls for the tolerogen-induced mice in Table 3.

Table 5. Specificities (number and percentage) of hybridomas derived from mice that received multiple immunizations of tomato spotted wilt virus (TSWV-BL or TSWV-OK)[a]

Experiments	Hybridoma specificities							
	BL-immunization				OK-immunization			
	Virus specific		Host specific		Virus specific		Host specific	
	Number	Percentage	Number	Percentage	Number	Percentage	Number	Percentage
I	5	3	135	97	3	4	68	96
II	7	8	81	92	9	12	65	88
III	5	3	186	97	7	6	103	94

[a] All adult mice were obtained from a supplier. Mice were immunized 3 times with either TSWV-BL or TSWV-OK preparation in experiments I and II, and 4 times in experiment III. Splenectomies were performed 4 days after the last immunization. Note, these are conventional procedures used for production of hybridomas.

Reaction of TSWV-MAB 1-7B4 in DAS-ELISA, PAS-ELISA and Dot-Immunobinding assay

DAS-ELISA was tested because of the widespread use of this assay. PAS-ELISA was tested because antibody does not need to be modified for this assay and because of the affinity of mouse immunoglobulin of the IgG2b subclass to protein-A. The DIBA was tested because the assay can be completed in a short period of time and has been shown to work with MAB to detect non-enveloped viruses (Sherwood et al., 1987).

For comparison of sensitivity of the three assays in detecting TSWV from different hosts, the samples were prepared as outlined in the Materials and Methods, dilutions made as appropriate, and the sample used in each of the three assays. For the DAS-ELISA and PAS-ELISA a reading was considered positive if the absorbance value at 405 nm was greater than the average absorbance value of the control plus three standard deviations. In the DIBA a sample was considered positive if a discernable difference in color between the sample and the control was evident on the NC sheet. In either DAS-ELISA or PAS-ELISA, regardless of the isolate and host, TSWV could be detected at a 1/2560 dilution after 30 min (Table 1). The TSWV-OK and TSWV-TN isolates could be detected at a 1/5120 dilution, the greatest dilution tried, with either ELISA. In the DIBA the greatest dilution that could be detected was 1/1280 with the TSWV-OK isolate in D. stramonium. The other isolates were detectable at dilutions of 1 or 2 fold less (Table 1). In addition to the isolates indicated in Table 1, the same assays were conducted on the TSWV-AR and TSWV-Type isolates maintained in D. stramonium, and the TSWV-I isolate maintained in N. benthamiana. The results for the TSWV-AR and TSWV-Type isolates were the same as for the TSWV-OK isolate maintained in D. stramonium. The TSWV-I isolate did not react with MAB 1-7B4 in any of the assays.

Storage of samples for detection of TSWV

The utility of the MAB 1-7B4 in diagnosis was further examined by looking at the reaction of samples stored in various fashions for different lengths of time. Samples of the TSWV-T1 and TSWV-T2 isolates in tomato, and the TSWV-TN in tobacco were used in this assay. Material was either ground in sample buffer or left intact before storing at 4 C or -20 C for different periods of time. Material ground prior to storage lost most of its serological reactivity regardless if it was stored at 4 C or -20 C. Virus could readily be detected in material left intact and stored at 4 C for 14 days regardless of the host, but much of the serological reactivity of the material stored intact at -20 C was lost.

Identification of antigen to TSWV-MAB

A number of attempts were made by Western blotting to determine which of the proteins of TSWV served as the homologous antigen for the MAB produced. However, when TSWV was denatured with SDS or several other ionic or nonionic detergents prior to electrophoresis the serological reactivity of the virus was lost.

When TSWV was disrupted according to Verkleij and Peters (1983), two fractions were obtained. One fraction contained the proteins of approximately 52 kDa, 58 kDa, and 78 kDa which are the glycoproteins. The other fraction contained only the 29 kDa fraction. When the fractions were run in DAS-ELISA, the MAB 1-7B4 reacted only to the fraction with the 29 kDa protein indicating the MAB reacts to the nucleocapsid protein (Table 2).

amounts of antibody for the diagnosis and study of TSWV. This technique should prove to be useful for other enveloped plant viruses.

In the research by the first author, during the initial screening for antibody production by the hybridomas a sandwich type assay had to be used. Attempts to bind the virus to the plastic wells with carbonate coating buffer were not successful. This may be applicable to other enveloped viruses. The MAB produced was useful in a variety of assays. The PAS-ELISA and dot-immunobinding assays did not require modification of the MAB. The limit of detection of TSWV was much less for the dot-immunobinding assay than the PAS-ELISA or DAS-ELISA. The assay of choice would depend on the requirements of the situation.

All isolates of TSWV except the TSWV-I isolate reacted to the MAB. This indicates that several isolates are serologically related even though they are from different hosts and geographical areas. The results of the DAS-ELISA with NP40 disrupted virions indicate the nucleocapsid protein of TSWV was the homologous antigen for MAB 1-7B4. This protein may be conserved between isolates of the virus just as is the nucleocapsid protein of isolates of many other viruses.

In the research by the second author, immunological tolerance to plant antigens was induced in mice by injecting newborn mice with plant extracts. Induction of immunological unresponsiveness in adult animals following injection neonatally with a tolerizing antigen has been previously described (Smith and Bridges 1958). Unlike immunosuppression, which is nonspecific, immunological tolerance is a form of specific responsiveness in which the animal's immune system responds in a negative way to a specific antigenic stimulation (Herbert and Wilkinson 1971). Tolerance is more readily established in the newborn than in adult animals (Cambier et al., 1977; Szewczuk and Siskind 1977). Induction of tolerance to plant antigens in mice before immunization with plant virus preparations offers a significant improvement over the standard methods of immunization in producing hybridomas to TSWV and possibly other plant viruses. The immune system of the mouse is still being developed in 5-wk-old mice. As a consequence, there was an increasing number of immunogen-specific (both TSWV and plant antigens) hybridomas derived from mice of increasing ages. Development of the immune system also resulted in increasing numbers of TSWV-specific hybridomas mice rendered immunologically tolerant to plant antigens in relation to the increasing age of mice at the time of immunization. Although not completely achieved in these experiments, a higher degree of tolerance was induced in 5-wk-old mice than in 7- or 9-wk-old mice and was successfully used in obtaining immune splenocytes for target antigens. The failure to induce complete tolerance is probably due to the heterogeneous nature of constituents in the plant extract (tolerogen preparations) in which some components may be present in suboptimal concentrations for induction of tolerance.

Immunological tolerance established in the neonatal period provides an extremely useful approach for the production of target specific hybridomas for antigens that are difficult to obtain in pure form. MAB to TSWV produced using immunotolerance or the more conventional approach will prove to be useful for developing rapid and reliable diagnostics for the virus.

REFERENCES

Alden, E. 1956. The production of an antiserum against an antigen from an unstable plant virus. M. S. thesis. University of Illinois, 37 pp.

Best, R. J. 1968. Tomato spotted wilt virus. Adv. Virus Res. 13:65-146.

Black, L. M., and Brakke, M. K. 1954. Serological reactions of a plant virus transmitted by leafhoppers. (Abstr.) Phytopathology 44:482.

Black, L. M., Brakke, M. K., and Vatter, A. E. 1963. Purification and electron microscopy of tomato spotted wilt virus. Virology 20:120-130.

Brakke, M. K. 1951. Density gradient centrifugation: a new separation technique. J. Am. Chem. Soc. 73:1847-1848.

Cambier, J. C., Vitetta, E. S., Uhr, J. W., and Kettman, J. R. 1977. B cell tolerance. II. Trinitrophenyl human gamma globulin-induced tolerance in adult and neonatal murine B cells responsive to thymus-dependent and independent forms of the same hapten. J. Exp. Med. 145:778-787.

Cho, J. J., Mau, R. F. L., Gonsalves, D., and Mitchell, W. C. 1986. Reservoir weed hosts of tomato spotted wilt virus. Plant Dis. 70:1014-1017.

Cho, J. J., Mau, R. F. L., Hamasaki, R. T., and Gonsalves, D. 1988. Detection of tomato spotted wilt virus in individual thrips by enzyme-linked immunosorbent assay. Phytopathology 78:1348-1352.

Clark, M. F., and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Edwards, M. L., and Cooper, J. I. 1985. Plant virus detection using a new form of indirect ELISA. J. Virol. Methods 11:309-319.

Felton, L. D., and Ottinger, B. 1942. Polysaccharide as a paralyzing agent on the mechanism of immunity in white mice. (Abstr.) J. Bacteriol. 43:94-95.

Francki, R. I. B., and Hatta, T. 1981. Tomato spotted wilt virus. Pates 491-512 in: Handbook of Plant Virus Infections and Comparative Diagnosis. E. Kurstak, ed. Elsevier-North Holland Biomedical Press, New York.

George, R. A., and Converse, R. H. 1988. Methods for the enrichment of desired B-cell populations before anti-cauliflower mosaic virus hybridoma formation. Phytopathology 78:1631-1636.

Gonsalves, D., and Trujillo, E. E. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.

Herbert, W. J., and Wilkinson, P. C. 1971. A Dictionary of Immunology. Blackwell Scientific Publications, Oxford and Edinburgh. 195 pp.

Hirata, A. A., Garvey, J. S., and Campbell, D. H. 1960. Retention of antigen in tissue of serologically suppressed chickens. J. Immunol. 84:576-581.

Hsu, H. T., Aebig, J., and Rochow, W. F. 1984. Differences among monoclonal antibodies to barley yellow dwarf viruses. Phytopathology 74:600-605.

Hsu, H. T., Franssen, J. M., van der Hulst. C. T. C., Derks, A. Fl L. M., and Lawson, R. H. 1988. Factors affecting selection of epitope specificity of monoclonal antibodies to tulip breaking potyvirus. Phytopathology 78:1337-1340.

Hsu, H. T., and Lawson, R. H. 1985. Comparison of mouse monoclonal antibodies and polyclonal antibodies of chicken egg yolk and rabbit for assay of carnation etched ring virus. Phytopathology 75:778-783.

Hsu, H. T., Wang, Y. C., Lawson, R. H., Wang, M., and Gonsalves, D. 1990. Splenocytes of mice with induced immunological tolerance to plant antigens for construction of hybridomas secreting tomato spotted wilt virus specific antibodies. Phytopathology 80:158-162.

Kohler, G., and Milstein, C. 1975. Continuous culture of fused cells secreting antibody of predefined specificity. nature 256:495-497.

Laemmli, U. K. 1970. Cleavage of structural proteins during the assembly of the head bacteriophage T4. Nature 227:680-685.

Leary, J. J., Brigati, D. J., and Ward, D. C. 1983. Rapid and sensitive colorimetric method visualizing biotin-labeled DNA probes hybridized to DNA or RNA immobilized on nitrocellulose: Bio-blots. Proc. Natl. Acad. Sci. 80:4045-4049.

Mohamed, N. A., Randles, J. W., and Francki, R. I. B. 1973. Protein composition of tomato spotted wilt virus. Virology 56:12-21.

Sherwood, J. L., Sanborn, M. R., and Keyser, G. C. 1987. Production of monoclonal antibodies to peanut mottle virus and their use in enzyme-linked immunosorbent assay and dot-immunobinding assay. Phytopathology 77:1158-1161.

Sherwood, J. L., Sanborn, M. R., Keyser, G. C., and Myers, L. D. 1989. Use of monoclonal antibodies in detection of tomato spotted wilt virus. Phytopathology 79:61-64.

Smith, R. T., and Bridges, R. A. 1958. Immunological unresponsiveness in rabbits produced by neonatal injection of defined antigens. J. Exp. Med. 108:227-250.

Szewczuk, M. R., and Siskind, G. W. 1977. Ontogeny of B-lymphocyte function. III. In vivo and in vitro studies on the ease of tolerance induction in B lymphocytes from fetal, neonatal and adult mice. J. Exp. Med. 145:1590-1601.

Verkleij, F.N., and Peters, D. 1983. Characterization of a defective form of tomato spotted wilt virus. J. Gen. Virol. 64:677-686.

CHARACTERIZATION OF MOUSE MONOCLONAL ANTIBODIES PREPARED TO A BULGARIAN ISOLATE OF TOMATO SPOTTED WILT VIRUS

G. Adam[1], D.-E. Lesemann[2], and H. J. Vetten[2]

Mouse hybridoma cell lines were produced by fusion of mouse myeloma cells (FOX-NY cell line) and spleen cells of a mouse immunized with a Bulgarian isolate of tomato spotted wilt virus (TSWV). Ten different stable hybridoma cell lines were obtained which secreted TSWV-specific monoclonal antibodies (MCA). These antibodies were screened against 11 different TSWV isolates. Among the 11 different isolates, a peanut isolate from South Africa could be clearly differentiated from the rest. Two MCA's 2B6 and 2G7, were present which did not show reduced reaction with this specific isolate and one MCA, 4F2, in addition revealed a more or less uniform reaction with all isolates. The structural proteins with which the available MCA's reacted were determined by triple antibody ELISA (TAS) tests with envelope and core protein fraction of purified TSWV. The two MCA's 2B6 and 2G7 were directed against an envelope protein, whereas the rest of the MCA's, including 4F2, reacted with the core protein.

INTRODUCTION

Tomato spotted wilt virus, the only member of the tomato spotted wilt virus group (Francki & Hatta, 1981) appears to be a member of the Bunyaviridae (Milne & Francki, 1984; De Haan et al., 1989; Elliott, 1990). Because the virus is transmitted by insects of the family Thripidae, it has an extremely broad host range, generally causes severe damage to infected plants, and is of great economic importance (Cho et al., 1989). This is true not only for countries where the virus is endemic in nature, but also in other areas where monocultures under glass with associated vector populations are threatened by this plant pathogen. Reports about TSWV damage in German greenhouses has increased interest in this virus. There is a demand for a fast, reliable and sensitive diagnostic procedure in our country (Lesemann & Dalchow, 1989).

Since TSWV is difficult to purify free of host components and has at least 4 different structural proteins (Tas et al. 1977b) with unknown diagnostic potential, we decided to exploit the benefits of monoclonal antibodies (Köhler & Milstein, 1975) in order to establish a diagnostic test system that is based on the ELISA procedure. Production, selection and characterization of monoclonal antibodies against TSWV is reported in this paper.

MATERIALS AND METHODS

The following isolates were used in our studies: L3 from Bulgarian tobacco; L1 from Bulgarian tobacco; G from Bulgarian tobacco; N1 from Alstromeria in the Netherlands; Ra from Ranunculus in Germany; Imp-1 from Impatiens in Mexico, Imp-2 from Impatiens imported to Germany; Pea from peas in South Africa; Pn from Peanuts in South Africa and Chry from Chrysanthemum imported to Germany. All TSWV isolates were maintained and propagated by mechanical inoculation on Nicotiana rustica L., Nicotiana benthamiana Domin., and Nicotiana glutinosa L., line A24.

Polyclonal TSWV-antisera from rabbits were kind donations from Dr. D. Gonsalves, New York State Agricultural Experiment Station, Geneva, New York, and Dr. D. Peters, Wageningen, the Netherlands. In addition, commercially available phytodiagnostic kits were

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1 Adam is head of the Plant Virus Group of the DSM-Deutsche Sammlung von Mikrooganismen und Zellkulturen GmbH.; Mascheroderweg 1b; D-3300 Braunschweig; FRG.

2 Lesemann is electron microscopist and Vetten is plant virologist at the Biologische Bundesanstalt Braunschweig Institut für Viruskrankheiten der Pflanze; Messeweg 11/12; D-3300 Braunschweig, FRG.

purchased from Agdia, Elkhart, IN, and Loewe Biochemicals. Goat anti-mouse antibodies, coupled with alkaline phosphatase, were obtained from Jackson Immunoresearch Laboratories, USA. Lectins were obtained from KEM-EN-TEC Denmark. Streptavidin alkaline phosphatase complexes were from DAKOPRATTS, Hamburg, FRG.

Virus purification

For the studies described in this paper only the L3 isolate was purified according to a procedure of Black et al. (1963) with modifications described by Tas et al. (1977a). Usually two successive sucrose gradient cycles were applied. The first was a sedimentation run and the second as an equilibrium separation. For fractionation of virus into cores and detergent-solubilized envelope proteins the virus band from the first sucrose gradient was treated for 1 hr at 4°C with 2% Triton X-100 and the cores sedimented by centrifugation (Beckman Ti 60 rotor, 2 hr. 40,000 rpm, 4°C).

Immunization

Polyclonal antiserum against TSWV was produced in a rabbit which received two intramuscular injections of 500 µl of purified TSWV emulsified in equal amounts of Freund's adjuvant 14 days apart. First bleedings were taken 1 week after the second injection. Serum samples were assayed on Western blots and in indirect DAS-ELISA tests for the presence of specific antibodies.

BALB/c mice were immunized by subcutaneous injections of 100-150 µl of purified TSWV emulsified in an equal volume of Freund's incomplete adjuvant. The same immunization procedure was repeated three and ten weeks after the first injection. After resting for four weeks, each mouse received a final booster by intraperitoneal injection of 100 µl purified virus.

Establishment, Selection and Cloning of Hybridoma Cells

Three days after the final booster a mouse was sacrificed and spleen cells were harvested and fused with the mouse myeloma cell line FOX-NY in the presence of 50% polyethylene glycol 4000 (Serva). Fused cells were resuspended in Dulbecco's modified eagle medium containing 1200 mM hypoxanthine, 0.4 mM aminopterin, 16 mM thymidine, 20% fetal calf serum and seeded into 96-well culture plates using syngeneic thymocites as feeder cells. Eleven days after the fusion, the supernatant fluids from the culture wells were screened for anti-TSWV antibody secretion by an indirect triple antibody sandwich ELISA (TAS-ELISA) against TSWV-infected and healthy plant extracts. For the ELISA tests we used the buffers described by Clark & Adams (1977).

Selected antibody secreting hybridomas were transferred to 24-well culture plates and retested. Positive cultures were cloned by the limiting dilution method. The immunoglobulins were typed using a similar ELISA procedure as described previously. However, instead of goat-anti-mouse antibodies coupled with alkaline phosphatase we used IgG isotype and IgM-specific goat antisera (Nordic) followed by a donkey anti-goat IgG alkaline phosphatase conjugate (Jackson Immunoresearch Laboratories, USA) and incubated for 2 hr at a dilution of 1:5000 in conjugate buffer.

RESULTS AND DISCUSSION

After selection and cloning, stable hybridoma cell lines were obtained (Table 1). Cell lines coded with the identical first three figures are sister clones. Typing of the antibodies indicated that the sister clones were identical since in each case they belonged to the same subtype of IgG. Only IgG-secreting hybridoma cell lines were obtained.

Table 1: TSWV-Monoclonal Antibodies: Specificity Tests With Different TSWV-Isolates

MCA Code	TSWV-ISOLATE Ra	G	Pn	Impl	L1	N1	P	L3	Imp2	Healthy	Max. Val.	Min. Val.	Average	STD
1B1-E4-G11	2.43	1.81	1.26	1.31	1.68	1.85	3.02	2.22	2.03	-0.01	3.02	1.25		
1B1-E4-F9	2.23	1.75	1.24	1.34	1.65	1.72	2.93	2.38	1.82	-0.00	2.93	1.24	1.93	0.51
1C7-H5	2.11	1.71	1.32	1.17	1.40	1.68	2.69	2.16	1.76	-0.00	2.69	1.17		
1C7-B4	2.37	1.90	1.30	1.30	1.66	1.71	2.83	2.31	1.99	0.01	2.83	1.30	1.85	0.47
2A8-F10	3.04	2.84	0.50	2.58	2.95	3.13	3.30	3.50	3.23	0.05	3.50	0.50		
2A8-F6	3.26	2.56	0.09	2.41	2.69	2.92	3.48	2.96	2.91	0.01	3.48	0.09	2.69	0.88
2B6-A2	3.05	2.21	2.26	1.37	2.44	2.41	3.07	2.84	1.18	0.01	3.07	1.18		
2B6-C6	3.15	2.02	2.51	1.33	2.54	2.58	2.92	2.94	0.94	0.01	3.15	0.94	2.32	0.67
2D12-D6-E2	2.36	1.78	1.11	1.51	1.74	1.96	2.98	2.42	2.41	-0.01	2.98	1.11		
2D12-B5	2.06	1.65	1.27	1.38	1.70	1.67	2.80	2.15	1.90	0.01	2.80	1.27	1.94	0.50
2G7-H9	2.93	1.48	2.16	1.54	2.46	2.43	2.92	2.62	1.05	0.01	2.93	1.05		
2G7-A11-C2	3.29	2.01	2.47	1.40	2.63	2.55	2.90	3.05	0.98	0.02	3.29	0.98	2.29	0.67
3E9-F2-F4	2.39	1.76	0.23	1.60	1.86	1.87	2.68	2.41	2.22	-0.02	2.68	0.23		
3E8-F2-C4	1.86	1.55	0.25	1.36	1.61	1.72	2.51	2.04	1.82	0.00	2.51	0.25	1.76	0.64
4F2-F9-A4	3.15	2.97	1.22	2.79	2.98	3.14	3.45	3.15	3.31	0.01	3.45	1.22		
4F2-D10	3.33	2.96	1.53	2.99	3.17	3.19	3.11	3.28	3.24	0.02	3.33	1.53	2.94	0.58
6G3-D8	2.21	1.61	0.84	1.31	1.66	1.81	2.49	2.16	1.00	-0.02	2.49	0.84		
6G3-A5	1.85	1.49	0.95	1.33	1.58	1.70	1.76	1.82	1.07	0.01	1.85	0.95	1.59	0.44
4H-E7-C9	1.48	1.35	0.92	1.08	1.63	1.67	1.44	1.57	1.49	0.01	1.67	0.92		
4H-E7-B8	1.27	1.13	0.86	1.13	1.61	1.71	1.10	1.43	1.38	0.01	1.71	0.38	1.29	0.33
Rabbit IgG	0.68	0.48	0.11	0.39	0.54	0.45	0.42	0.62	0.08	0.00	0.68			

The rabbit IgG was obtained from D. Peters, Wageningen. Reactions were measured after an incubation time of 20 min at 37°C. Freeze dried sap from N. rustica infected with TSWV L3 was used as an antigen after rehydration with ELISA-sample buffer at a final dilution of 1:240.

The reactions of the different secreted IgG's with 9 different TSWV isolates are summarized in Table 1. The Chrysanthemum isolate was not available at the time of testing but it reacted in later tests like the N1 isolate. As can be seen from the results in Table 1, the lowest ELISA values were obtained in most cases with the isolate Pn, followed by the isolate Imp 2, which differed from the isolate Imp 1 by its symptom expression on Impatiens where it caused severe ringspots. The other isolates, especially from tobacco, reacted more or less uniformly with the panel of available MCA's.

Since we were mainly interested in antibodies with a broad reactivity and high sensitivity, we have chosen for further studies those MCA's with a high average and a low standard deviation calculated from their reaction against all TSWV isolates. This led to the selection of the MCA's 2B6, 2G7 and 4F2 for application in ELISA test systems.

MCA's were screened for their capacity to react with complete TSWV particles in the electron microscope. Since only MCA's directed against the surface structural proteins of the virions appeared to be applicable, it was necessary to determine the specificity of the MCA's to structural proteins of the virus. Preliminary attempts to screen the MCA's on Western blots were unsuccessful.

None of the MCA's reacted with the structural proteins after SDS denaturation, gel electrophoresis and subsequent transfer to nitrocellulose. We, therefore, decided to solubilize the envelope proteins by detergent treatment and separate them from the stable cores by ultracentrifugation. The two fractions were then used in parallel as antigens in TAS ELISA with our MCA's. The results of these experiments are summarized in Table 2. Among the 10 different MCA's, two, 2B6 and 2G7 clearly reacted with the envelope protein containing supernatant fraction. The others were either clearly directed against the core fraction or of ambiguous reaction. Electrophoretic analyses of the two fractions indicated that both fractions were contaminated with either core- or envelope-associated protein, respectively, which could explain the ambiguous results. Later tests, where envelope proteins were further purified chromatographically, indicated that only the MCA's 2B6 and 2G7 were directed against envelope proteins (results not shown). The results were substantiated in tests with the electron microscope, where only the two MCA's 2B6 and 2G7 bound to particles that appeared morphologically as complete TSWV particles (Adam et al., in preparation).

Our results are in good agreement with previously published results using MCA's against TSWV by Sherwood et al. (1989) and Huguenot (1989). However, all of our MCA's did react with purified virus and with extracts of infected plants, which is in contrast to the results described by Huguenot (1989) who observed with some of the MCA's positive reactions only when using plant extracts. The application of freeze-dried plant sap as the antigen for selection of the MCA's may explain this different result. The non-reactivity of our MCA's in Western blots was surprising but seems to be specific for the TSWV proteins as has also been observed by Huguenot (1989).

The high reactivity of core-specific antibodies in ELISA tests, especially MCA 4F2 as shown in Table 1, is in accordance with the results published in Huguenot (1989). However, the much better reaction of envelope specific MCA's (Table 1) with the Pn isolate indicated that core proteins are not as conserved among different isolates of TSWV as should be anticipated from results published for animal Bunyaviridae (Elliott, 1990). It appears that envelope proteins between different TSWV isolates are more closely serologically related as reported by Law and Moyers (1989) from their comparisons of a lettuce isolate with a new serotype from Impatiens.

Table 2: TSWV-Monoclonal Antibodies: Specificity Tests with Dissociated TSWV-L3.

MCA Code	Fraction and Dilution: Supern. 1:10	Supern. 1:100	Pellet 1:10	Pellet 1:100	Max. Val.	Max. Val.	Specificity
1B1-E4-G11	0.62	0.63	1.56	0.64	1.56	0.62	Core
1B1-E4-F9	0.79	0.54	1.77	1.04	1.77	0.54	Core
1C7-H5	0.75	0.51	1.68	0.90	1.68	0.51	Unclear
1C7-B4	0.81	0.56	1.64	0.78	1.64	0.56	Unclear
2A8-F10	1.67	1.19	2.66	1.47	2.66	1.19	Core
2A8-F6	1.57	1.00	2.57	1.43	2.57	1.00	Core
2B6-C6	0.67	1.49	0.81	0.21	0.81	0.21	Envelope
2D12-D6-E2	0.09	0.05	1.47	0.11	1.47	0.05	Core
2D12-B5	0.73	0.54	1.91	1.08	1.91	0.54	Core
2G7-H9	0.58	0.51	0.83	0.19	0.83	0.19	Envelope
2G7-A11-C2	0.67	0.53	0.79	0.19	0.79	0.19	Envelope
3E8-F2-F4	1.03	0.66	1.64	0.89	1.64	0.66	Unclear
3E8-F2-C4	0.97	0.67	1.67	0.94	1.67	0.67	Unclear
4F2-F9-A4	1.95	1.51	2.89	2.13	2.89	1.51	Core
4F2-D10	2.31	1.67	3.24	2.60	3.24	1.67	Core
6G3-D8	0.02	0.02	1.27	0.05	1.27	0.02	Core
6G3-A5	0.67	0.49	1.61	1.12	1.61	0.49	Core
4H9-E7-C9	0.79	0.50	1.05	1.03	1.05	0.50	Core
4H9-E7-B8	0.82	0.54	1.28	0.87	1.28	0.54	Core
Rabbit IgG	0.41	0.20	0.90	0.67	0.90	0.20	

Supernatant is the Triton X-100 solubilized fraction of purified TSWV, containing predominantly the envelope proteins.
Pellets are the TSWV cores containing predominantly the 28 kd N protein and the RNA. ELISA conditions were as described in Table 1.

From the results in Table 1 and 2 we have chosen the MCA 4F2 for use in an ELISA test system. Since the hybridoma cell lines did not produce sufficient quantities of ascites fluid in pristane primed mice, we have propagated the cells in larger amounts in spinner flasks and purified the MCA from the culture medium by ammonium sulfate precipitation and affinity chromatography on Protein A Sepharose (Pharmacia). The antibodies obtained were either labelled with alkaline phosphatase using the glutaraldehyde method, or with biotin-hydroxysuccinimidyl ester. These antibodies were used in different combinations as shown in Fig. 1 for ELISA tests. It is evident from Fig. 1 that the MCA 4F2 could be used as a trapping antibody on the microtiter plates and did not lose its reactivity by the two different derivatization procedures employed. In all cases the reactions with healthy plant sap were almost zero. From the results in Fig. 1 and other tests, and because of simpler handling in practice, we decided to use the combination of coating the plates with polyclonal antiserum and detection of bound antigen with the MCA labelled with alkaline phosphatase, despite the higher sensitivity that was obtained in indirect tests using goat anti-mouse alkaline phosphatase for the detection. When compared to ELISA-tests using polyclonal antiserum against TSWV, the detection system with our MCA was not only more sensitive and revealed lower reactions with healthy plant sap, but in addition readings could be taken after a much shorter time (results not shown). The sensitivitity that could be obtained with our system is demonstrated in Fig. 2, where a dilution series of infected and healthy plant sap was applied. The response is fairly linear from a dilution of 1:50 up to 1:1000. Since no reactions were obtained with the healthy controls, an extrapolation to further dilutions might be used to estimate a dilution of at least 1:5000 as a safe detection limit. This value is well within the range given by Huguenot (1989) and Sherwood *et al.* (1989).

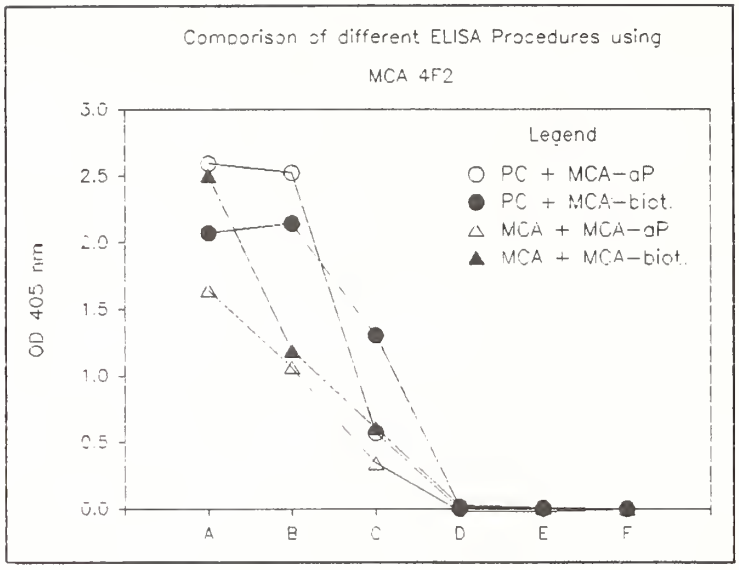

Figure 1. The plates were coated with 2 ug/ml IgG (PC = polyclonal; MCA = monoclonal). Antigens were TSWV L3-infected and healthy plant sap. The dilution is based on the weight of the plant tissue. Plates were measured after 45 min at 37°C. A = antigen 1:10; G = antigen 1:100; C= antigen 1:1000; D = healthy control 1:10; E = healthy control 1:100; F = Buffer.

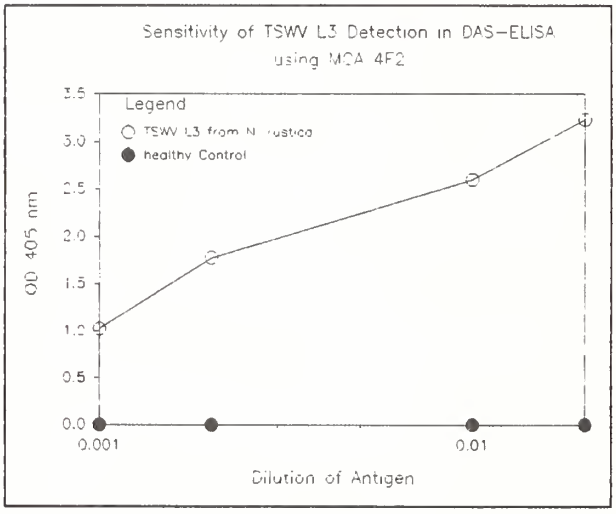

Figure 2. The ELISA test was performed as described in Fig. 1

In summary, our results show that monoclonal antibodies against TSWV in combination with polyclonal antibodies can be used successfully for the detection of the virus in infected plants with a high sensitivity and reliability. It remains to be determined, however, whether all different serotypes of TSWV can be detected with one single MCA or whether at least two are necessary to make the test generally applicable.

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REFERENCES

Black, L. M., Brakke, M. K. and Vatter, A. E. 1963. Purification and electron microscopy of tomato spotted wilt virus. Virology 20:120-130.

Cho, J. J., Mau, R. F. L., German, T. L., Hartman, R. W., Yidin, L. S., Gonsalves, D. and Provvidenti, R. 1989. A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. Plant Disease 73:375-383.

Clark, M. F. and Adams, A. N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virology 34:475-483.

De Haan, P., Wagemakers, L., Goldbach, R. and Peters, D. 1989. Tomato spotted wilt virus, a new member of the Bunyaviridae? In: Kolakofski and Mahy (eds.). Genetics and Pathogenicity of Negative Strand Viruses. Elsevier north Holland, pp. 287-291.

Elliott, R. M. 1990. Molecular biology of the Bunyaviridae. J. Gen. Virology 71:501-522.

Francki, R. I. B. and Hatta, T. 1981. Tomato spotted wilt virus. In: Handbook of Plant Virus Infections and Comparative Diagnosis. E.Kurstak (ed.). Elsevier, New York, pp. 491-512.

Huguenot, C. 1989. Etude immunochimique de deux virus des plantes: Le "Tomato Spotted Wilt Virus" et le "Peanut Clump Virus". Universite Louis Pasteur, Strasbourg.

Köhler, G. and Milstein, C. 1975. Continuous culture of fused cells secreting antibody of predefined specificity. Nature 256:495-497.

Law, M. D. and Moyer, J. W. 1989. Physiochemical analysis of a serologically distinct tomato spotted wilt virus strain. Phytopathology 79:1157.

Lesemann, D.-E. and Dalchow, J. 1989. Tomatenbronzefleckenvirus. Gärtner-börse Gartenwelt. 90:216-222.

Milne, R. G. and Francki, R.I.B. 1984. Should tomato spotted wilt virus be considered as a possible member of the family Bunyaviridae? Intervirology 22:72-76.

Sherwood, J. L., Sanborn, M. R., Keyser, G. C. and Myers, L. D. 1989. Use of monoclonal antibodies in detection of tomato spotted wilt virus. Phytopathology 79:61-64.

Tas, P.W.L., Boerjan, M. L. and Peters, D. 1977a. Purification and serological analysis of tomato spotted wilt virus. J. Gen. Virology 36:61-72.

Tas, P.W.L., Boerjan, M. L. and Peters, D. 1977b. The structural proteins of tomato spotted wilt virus. J. Gen. Virology 36:267-279.

DETECTION OF TOMATO SPOTTED WILT VIRUS BY ENZYME-LINKED IMMUNOSORBENT ASSAY, DOT-BLOT IMMUNOASSAY AND DIRECT TISSUE BLOTTING

H. T. Hsu and R. H. Lawson

ABSTRACT

Immunological analysis by direct tissue blotting was compared with enzyme-linked immunosorbent assay (ELISA) and a dot-blot immunoassay (DBIA) for detection of tomato spotted wilt virus (TSWV) in infected plants. TSWV was readily detected in tissue blots of infected Nicotiana benthamiana leaves. Using biotinylated mouse monoclonal antibodies, DBIA was nearly 8 times more sensitive than ELISA for detection of TSWV in extracts from infected N. benthamiana leaves. Leaf and stem samples from Eustoma grandiflorum (lisianthus) and several Impatiens plants showing virus-like symptoms tested positively by double antibody sandwich ELISA and by direct tissue blottings. Asymptomatic leaves or stems from the same plants tested negatively by ELISA. Although tissue blots of asymptomatic leaves and stems were negative to the naked eye, examination of the blots at 10-20X magnification under a dissecting microscope revealed positive reactions.

INTRODUCTION

Serological test methods widely used in plant virology include enzyme-linked immunosorbent assay (ELISA), dot-blot immunoassay (DBIA) and immune-specific electron microscopy (ISEM) (Clark and Adams, 1977; Derrick, 1973; Milne and Luisoni, 1975; Powell, 1987). Although extremely sensitive, ISEM is still essentially a research tool since electron microscopes are often not available for routine diagnosis.

ELISA and DBIA methods for plant virus detection depend on two assumptions. Viral antigens or antibody molecules must attach to a solid-phase substrate where they retain antigenicity. In addition, antibodies must be linked to an enzyme with both antibody and enzyme retaining activity. Experience has shown that both assumptions are true in assays of plant viruses. Antibody molecules and plant virus antigens retain activity although they attach to several different types of supports, including filter paper, nitrocellulose or nylon membranes and plastic surfaces such as polyvinyl chloride or polystyrene (Clark and Bar Joseph, 1984; Haber and Knapen, 1989; Scott, 1989). Antibodies have been linked to a variety of enzymes such as β-galactosidase, urease, horseradish peroxidase and alkaline phosphatase yielding stable, highly reactive reagents (Chandler et al., 1982; Moran et al., 1985; Neurath and Strick, 1981).

Both ELISA and DBIA require extraction of viral antigens into a suitable buffer. Direct tissue blotting requires minimal preparation of tissue samples for detection of plant viruses and mycoplasma-like organisms. (Lin et al., 1990). In this paper we compare tissue-blotting with ELISA and DBIA for detection of tomato spotted wilt virus (TSWV) in infected plants.

MATERIALS AND METHODS

Virus Cultures

Isolates of TSWV were obtained from several sources (Table 1). All isolates, except Mojave, were propagated in Nicotiana benthamiana L. by inoculating healthy plants with TSWV-infected leaves prepared in a buffered solution containing 0.033 M KH_2PO_4, 0.067 M K_2HPO_4, 0.01 M Na_2SO_3, pH 7.2 (Black et al., 1963). Several TSWV-infected Impatiens plants and a lisianthus plant were obtained from Costa Rica. All TSWV isolates were received under a permit issued by U.S. Department of Agriculture, Animal and Plant Health Inspection Service.

Hsu is a Microbiologist and Lawson is a Research Plant Pathologist, U. S. Department of Agriculture, Agricultural Research Service, Florist and Nursery Crops Laboratory, Beltsville Agricultural Research Center, Beltsville, Maryland, 20705.

Table 1. Sources of tomato spotted wilt virus and their propagation hosts.

Isolate	Propagation Host	Source
Impatiens	N. benthamiana	USDA[a], Beltsville, H.T. Hsu
Lettuce	N. benthamiana	Maui, Hawaii, H.T. Hsu
Mojave	Impatiens cv. Mojave	USDA, Beltsville, R.H. Lawson
Tomato-Can.	N. benthamiana	Agri. Canada[b], Ontario, J.A. Matteoni
Tomato-MD	N. benthamiana	MDA[c], Annapolis, M. Putnum

a. U.S. Department of Agriculture, Beltsville Agricultural Research Center
b. Agriculture Canada, Research Station, Vineland Station
c. Maryland Department of Agriculture

Immunological Reagents

Mouse monoclonal antibodies to TSWV were produced at the Florist and Nursery Crops Laboratory (Hsu et al., 1990). IgG2a mouse monoclonal antibodies were purified from ascitic fluids by column chromatography through protein A-sepharose (Hsu et al., 1982). Biotinylated monoclonal antibodies were used at 1 ng/ml antibody concentration (Hsu and Lawson, 1989). Avidin-alkaline phosphatase conjugate (Sigma Chemical, St. Louis, MO) was used at a 1/4,000 dilution. Rabbit polyclonal antisera and alkaline phosphatase-labelled or horseradish peroxidase-labelled rabbit antibodies to TSWV-L and a distinct isolate of TSWV (TSWV-I) (Law and Moyer, 1990) were obtained from Agdia, Inc. (Elkhart, IN) and were used at 1/1,000 dilutions. Alkaline phosphatase-labelled goat anti-mouse immunoglobulins and alkaline phosphatase-labelled goat anti-rabbit immunoglobulins (Kirkegaard and Perry Laboratory, Inc., Gaithersburg, MD) were reconstituted according to the manufacturer's direction, and were used at 1/1,000 dilutions.

Preparation of Tissue Extracts

One part of plant tissue was triturated in 19 parts of the buffered inoculum solution in a mortar with a pestle. Extracts were filtered through a layer of Miracloth (Calbiochem Corporation, San Diego, CA) before being transferred to test tubes. Further dilutions were made in a phosphate buffered saline solution (PBS, 0.02 M K_2PO_4, 0.15 M NaCl, pH 7.4).

Avidin-Biotin Mediated ELISA

Polyvinyl chloride plates were coated with 150 µl of rabbit polyclonal antibodies or mouse monoclonal antibodies (1 µg/ml in 0.1 M carbonate solution, pH 9.6) for 1-2 hr at room temperature. Following one washing with PBS-Tween (PBS containing 0.05% Tween 20), plates were blocked with 1% bovine serum albumin (BSA) in PBS-Tween for 1 hr at room temperature. Various dilutions of extracts from TSWV-infected and healthy control leaf tissues were then incubated in triplicates at room temperature for 2 hr or 4°C overnight. After 3 washings, 3 min each, with PBS-Tween, 150 µl biotinylated mouse monoclonal antibodies were added to each well and incubated at room temperature for 2 hr before the next washing with PBS-Tween and incubation with avidin-alkaline phosphatase conjugate at room temperature for an additional 2 hr. After washing with PBS-Tween, substrate solution, p-nitrophenyl phosphate at 1 mg/ml in 10% diethanolamine, pH 9.8, was added to each well. Color intensities were measured in an MR700 Dynatech ELISA Reader (Dynatech Laboratories, Inc., Chantilly, VA).

Double-Sandwich-Antibody (DAS-) ELISA

The procedure used in DAS-ELISA was similar to that in avidin-biotin mediated ELISA except that after the addition of antigens, alkaline phosphatase-labelled rabbit anti-TSWV immunoglobulins were used to detect the virus antigens. For horseradish peroxidase-labelled rabbit anti-TSWV immunoglobulins, O-phenylenediamine was used as a substrate.

Sample Application in DBIA

Nitrocellulose (NC) membranes, 0.45 μ pore size, (Schleicher & Schuell, Inc., Keen, NH) were used in DBIA. Samples (50 μl) were applied to previously wet NC membranes with the aid of a minifold apparatus (Schleicher & Schuell, Inc.) under vacuum. Each well was washed with 100 μl PBS before removing the NC membrane from the apparatus.

Tissue Blotting on NC Membranes

Sections were cut from fresh tissues by hand with a new razor blade for each sample. Leaves were first rolled into a tight core before cutting. Tissues were held in one hand and cut with a steady motion with the other hand to obtain a single plane cut surface. Tissue blots were made by pressing, with a firm but gentle force, the newly cut surface onto a 0.45 μ pore size NC membrane.

Detection of TSWV Antigens on NC Membranes

Dot blots or tissue blots were first immersed in PBS containing 1% BSA for 60 min with gentle shaking at room temperature followed by a brief rinse in PBS-Tween. For direct immunological detection, the blots were incubated for 60 min at room temperature with alkaline phosphatase-labelled TSWV-specific antibodies diluted in PBS. The blots were then washed three times by gentle shaking in PBS-Tween, 10 min each time before incubation in an enzyme substrate solution. In indirect immunological detection, NC membranes with dot blots or tissue blots were incubated with TSWV specific primary antibodies (rabbit polyclonal antisera or mouse monoclonal antibodies) diluted in PBS at room temperature for 60 min. Following three washings in PBS-Tween, the blots were reacted with enzyme-labelled species specific secondary antibodies (goat anti-rabbit immunoglobulins for rabbit antisera or goat anti-mouse immunoglobulins for mouse antibodies) for an additional 60 min. Before the addition of substrate, the blots were washed again three times in PBS-Tween with gentle shaking. In tests where the avidin-biotin system was employed, the blots were incubated in TSWV-specific biotinylated antibodies followed by reaction with avidin-alkaline phosphatase conjugates.

For color development, the blots were immersed in a solution containing 14 mg nitroblue tetrazolium and 7 mg 5-bromo-4-chloro-3-indolyl phosphate in 40 ml substrate buffer of 0.1 M Tris, 0.1 M NaCl, 5 mM $MgCl_2$, pH 9.5. A positive result is indicated by the development of a purple color on the blots. A negative reaction developed no color. The reactions were rated by direct observation of the membrane and in some cases, with an aid of a dissecting microscope.

RESULTS

Trapping Antibodies

The efficiency of mouse monoclonal antibodies and rabbit polyclonal antibodies for trapping TSWV antigens were compared in two different ELISA formats. The absorbance values of monoclonal antibody-coated wells were about two times those of polyclonal antibody-coated wells in indirect ELISA using biotinylated-mouse monoclonal antibodies with avidin-enzyme conjugates (Fig. 1A). In direct ELISA using enzyme-labelled TSWV specific rabbit polyclonal antibodies, the absorbance values of monoclonal antibody-coated wells were slightly lower than those of polyclonal antibody-coated wells (Fig. 1B).

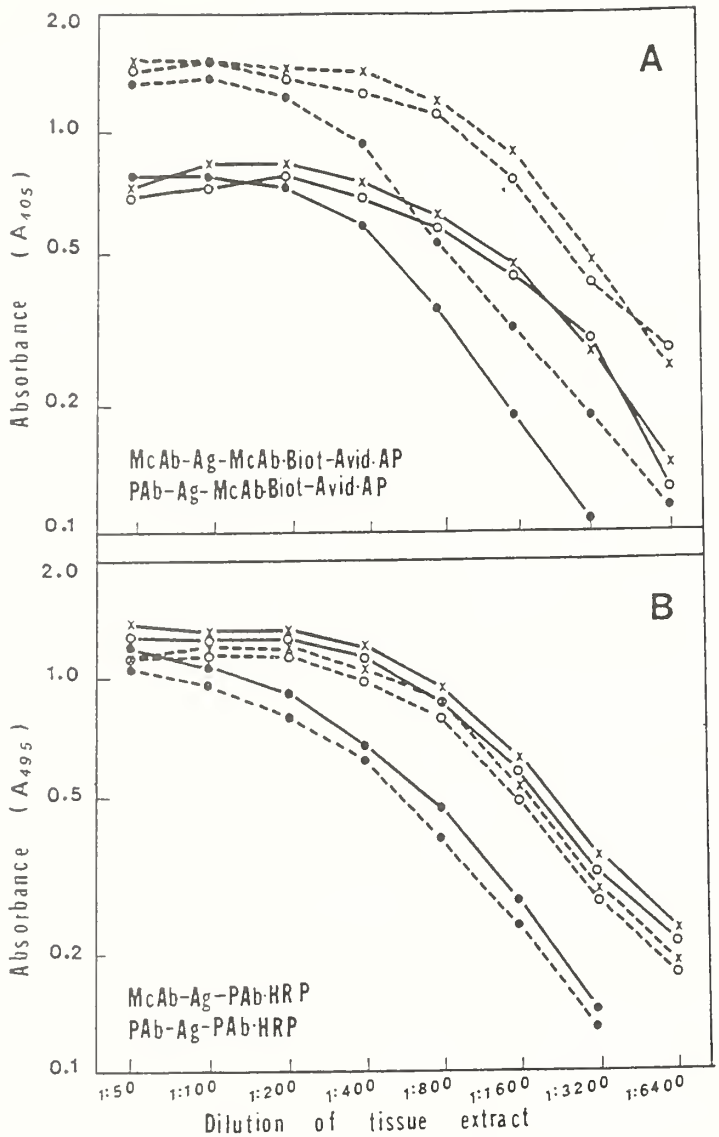

Figure 1. ELISA detection of tomato spotted wilt virus (TSWV) antigens (lettuce isolate, x; tomato-MD isolate, o; and tomato-Can isolate, •) in extracts of infected N. benthamiana leaves. TSWV antigens were trapped by either mouse monoclonal antibodies (dotted lines) or rabbit polyclonal antibodies (solid lines). Biotinylated mouse monoclonal antibodies and avidin-alkaline phosphatase conjugates (A), or horseradish peroxidase-labelled rabbit anti-TSWV immunoglobulins (B) were used for detection of TSWV.

TSWV Detection by DBIA and ELISA

Dilution endpoints of TSWV antigens in infected leaf tissues were determined by DBIA and ELISA. The DBIA dilution endpoint of the extract of TSWV-infected N. benthamiana leaf tissues was about $10^{-4.6}$ when biotinylated mouse monoclonal antibodies and avidin-enzyme conjugates were used and was about 10^{-4} when unlabelled mouse monoclonal antibodies and an enzyme-labelled goat anti-mouse immunoglobulin conjugate was employed. Using biotinylated monoclonal antibodies and avidin-alkaline phosphatase conjugates in mouse monoclonal antibody coated ELISA plates, the dilution endpoint of the same infected leaf extracts was about $10^{-3.7}$.

TSWV Detection by Tissue Blotting

TSWV was readily detected in tissue blots from infected leaves. The presence of TSWV antigens in blots of infected tissues is evidenced by the development of intense purple color, whereas the control healthy leaf blots did not develop purple color but retained the green color of chlorophyll. TSWV antigens were identified from tissue blots of N. benthamiana infected with lettuce, tomato-MD or tomato-Can isolates by a rabbit polyclonal antiserum or mouse monoclonal antibodies prepared to TSWV-L; whereas virus antigens could be detected in tissue blots of N. benthamiana leaves infected with an Impatiens isolate when tested with TSWV-I rabbit polyclonal antiserum. Detection of TSWV from infected Impatiens cv. Mojave was unsuccessful due to a high concentration of colored pigments in the tissue blots.

Comparison of ELISA and Tissue Blottings for TSWV Detection

One lisianthus plant and samples of symptomatic and asymptomatic leaves/stems from several Impatiens plants with suspected TSWV infections were obtained from Costa Rica and tested by both ELISA and direct tissue blottings. All samples bearing virus-like and necrotic symptoms tested positively at 1/100 dilutions by DAS-ELISA, 1/20 dilution (Table 2). Although tissue blottings of asymptomatic leaves/stems were negative to the naked eye, careful examination of tissue blots at 10-20X magnification under a dissecting scope revealed the presence of positive reactions (Table 2). All samples tested negatively by both ELISA and tissue blottings when monoclonal antibodies prepared to a lettuce isolate of TSWV were used.

Table 2. Detection of TSWV antigens by enzyme-linked immunosorbent assay and direct tissue blotting.

Plant	Tissue/Source	Blots		ELISA	
		Visual	Microscopical	1/20	1/100
67-1 (#11)	Symptomatic leaf	+	+	1.16	0.99
Impatiens	Asymptomatic leaf	-	+	0	0
	Necrotic stem	+	+	0.83	0.46
67-1 (#11d)	Symptomatic leaf	+	+	1.1	0.81
Impatiens	Asymptomatic leaf	-	+	0.02	0
	Necrotic stem	+	+	0.53	0.2
1088-3 (# 22)	Symptomatic leaf	+	+	1.0	0.84
Impatiens	Asymptomatic leaf	-	+	0	0
1088-3 (#22a)	Symptomatic leaf	+	+	1.1	0.95
Impatiens	Asymptomatic leaf	-	+	0	0
WRG 163-C5 (#13)	Symptomatic leaf	-	-	0	0
Impatiens	Asymptomatic leaf	-	-	0	0
L13C491-11-1-4-5 Lisianthus		+	+	1.2	1.1
Positive control		+	+	1.2	0
Negative control		-	-	0	0

DISCUSSION

Both ELISA and DBIA are routinely used for detection of plant viruses and diagnosis of infection. Our results show that higher dilution endpoints of TSWV antigens in extracts of infected leaf tissues were, however, demonstrated by DBIA than by ELISA. This may be due to the deposition of more TSWV antigens on NC membranes than in PVC ELISA plates. Tests with several other viruses indicated that only about 25-37% of antigens applied to each well were actually trapped by coated specific antibodies in ELISA plates (Hsu and Aebig, unpublished data). In addition only a limited small sample volume, usually no more than 200 1, can be added to each well. For immunoassays of viruses on NC membranes, binding of antigens to solid support is efficient. In DBIA, TSWV antigens were detected only on the side of the membrane where the samples were applied. No antigens were detected on the opposite side. Complete or near complete estimates of viral antigens in dilute sample solutions on NC membranes is possible. It was estimated that a protein binding capacity of 80 g/cm^2 was reported for NC membranes (Gershoni and Palade, 1982). Furthermore, a volume larger than 200 1 can be applied since virus antigens are collected and bound to NC matrix under vacuum.

Viral antigens can be visually localized with enzyme-labelled antibodies in tissue blots on NC membranes. In addition to TSWV, immunological analysis of plant virus antigens by direct tissue blottings has been described for several viruses in cucumovirus, luteovirus, potexvirus and potyvirus (Lin et al., 1990). The presence of specific antigens is characterized by the development of purple color in the tissue blots on membranes. Localization of specific antigens such as a luteovirus and a mycoplasma-like organism in specialized tissue (phloem) also has been clearly demonstrated, (Lin et al., 1990).

Establishment of dilution endpoint of infected tissues by direct tissue blotting of NC substrate is difficult. Since NC membranes provide an excellent substrate for assays of TSWV antigens by DBIA, we anticipate similar sensitivities would be possible. The positive reactions that escape examination by the naked eye that can be identified under a dissecting microscope further increase the usefulness of tissue blotting for virus detection. Extracts of mildly symptomatic tissue where only a few cells are infected with a virus and the virus is irregularly distributed, may give only a weak signal or may be undetected by ELISA.

The tissue blotting technique for detection of TSWV antigens from infected tissue demonstrates the basic value of immunological detection of plant virus antigens with the methods described in here. In addition to the advantages of specificity, sensitivity, reliability and rapidity that ELISA and DBIA offer, the direct tissue blotting technique also provides simplicity and convenience for the assay of larger numbers of samples.

REFERENCES

Black, L.M., Brakke, M.K., and Vatter, A.E. 1963. Purification and electron microscopy of tomato spotted wilt virus. Virology 20:120-130.

Chandler, H.M., Cox, J.C., Healey, K., MacGregor, Al., Premier, R.R., and Hurrell, J.G.R. 1982. An investigation of the use of urease-antibody conjugates in enzyme-immunoassays. J. Immunol. Methods 53:187-194.

Clark, M.F., and Adams, A.N. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Derrick, K.S. 1973. Quantitative assay for plant viruses using serologically specific electron microscopy. Virology 56:652-653.

Gershoni, J.M., and Palade, G.E. 1982. Electrophoretic transfer of proteins from sodium dodecyl sulphate polyacrylamide gels to a positively charged membrane filter. Anal. Biochem. 124:396-405.

Haber, S., and Knapen, H. 1989. Filter paper sero-assay (FIPSA): A rapid, sensitive technique for sero-diagnosis of plant viruses. Can. J. Plant Pathol. 11:109-113.

Hsu, H.T., Aebig, J., Civerolo, E.L., and Helkie, C. 1982. Enzyme conjugated protein A: A universal reagent for indirect enzyme-linked immunosorbent assays (ELISA). Phytopathology 72:938.

Hsu, H.T., and Lawson, R.H. 1989. Detection of tomato spotted wilt virus in Impatiens using biotinylated mouse monoclonal antibodies. Phytopathology 79:1197.

Hsu, H.T., Wang, Y.C., Lawson, R.H., Wang, M., and Gonsalves, D. 1990. Splenocytes of mice with induced immunological tolerance to plant antigens for construction of hybridomas secreting tomato spotted wilt virus-specific antibodies. Phytopathology 80:158-162.

Law, M.D., and Moyer, J.W. 1990. A tomato spotted wilt-like virus with a serologically distinct N protein. J. Gen. Virol. 71:933-938.

Lin, N.S., Hsu, Y.H. and Hsu, H.T. 1990. Immunological detection of plant viruses and a mycoplasma-like organism by direct tissue blotting on nitrocellulose membranes. Phytopathology 80:824-828.

Milne, R.G., and Luisoni, E. 1975. Rapid high resolution immune electron microscopy of plant viruses. Virology 68:270-274.

Moran, J.R., Wilson, J.M., Garrett, R.G., and Smith, P.R. 1985. ELISA indexing of commercial carnations for carnation mottle virus using a unrease-antibody conjugate. Plant Pathol. 34:467-471.

Neurath, A.R., and Strick, N. 1981. Enzyme-linked fluorescence immunoassays using β-galactosidase and antibodies covalently bound to polystyrene plates. J. Virol. Methods 3:155-165.

Power, C.A. 1987. Detection of three plant viruses by dot-immunobinding assay. Phytopathology 77:306-309.

Scott, D.I. 1989. Immunoblotting and dot blotting. J. Immunol. Methods 119:153-187.

TOMATO SPOTTED WILT VIRUS AND ONE THRIPS VECTOR: *Frankliniella occidentalis* (Pergande) INTERNAL MORPHOLOGY AND VIRUS LOCATION

Ullman, D.E., Westcot, D.M., Hunter, W.B., Mau, R.F.L., Cho, J.J. and D. Custer

ABSTRACT

Internal anatomy and morphology of adult western flower thrips (WFT), *Frankliniella occidentalis* (Pergande) differs from many other species previously studied in number, position and ducting of the salivary glands, the morphology of the alimentary canal and the number and arrangement of the malphighian tubules. Larval and adult WFT morphology are similar, although looping of the midgut in larvae differs from that observed in adults. Using immunology and transmission electron microscopy (TEM), tomato spotted wilt virus (TSWV) has been located in the midgut epithelial cells of larvae and adults, a specialized fat body aligned with the gut and salivary glands and the salivary glands of WFT that have acquired the virus. Crystalline arrays, similar to those frequently associated with replicating viruses, have been observed in WFT fat body although their association with TSWV has not yet been definitively demonstrated. The significance of viral location in various tissues and presence of crystalline arrays is discussed.

INTRODUCTION

Thrips (Order: Thysanoptera) are diminutive insects that are widespread throughout the world in habitats ranging from forests, grasslands and scrub to cultivated crops and gardens (Lewis, 1973). Among the 5,000 described Thysanopteran species (zur Strassen, 1960) there are only six, *Frankliniella occidentalis* (Pergande), *F. schultzei* (Trybom), *F. fusca* (Hinds), *Scirtothrips dorsalis* (Hood), *Thrips tabaci* Lindeman and *T. setosus* Moulton, that are known to transmit tomato spotted wilt virus (TSWV) (Amin, 1981; Gardner et al, 1935; Kobatake, 1984; Sakimura, 1963; Samuel, et al, 1930). Recent unpublished work on watermelon and groundnuts suggests that a seventh species, *T. palmi*, may also transmit certain TSWV strains (Chen and Whitman, respectively, pers. comm.).

Thrips are unique among all insect vectors of plant viruses in that they must acquire TSWV as larvae in order to transmit the virus. Adults feeding for the first time on a virus source never become inoculative (Bald and Samuel, 1931; Sakimura, 1962). The underlying mechanisms governing this phenomenon and specificity between TSWV and only a few thrips species are not yet understood. Furthermore, many questions regarding acquisition, persistence, replication, and location of TSWV within thrips and how these factors impact transmission efficiency remain unanswered (Cho, et. al., this proceedings). Our approach to answering some of these questions has been to develop basic information regarding thrips/TSWV interactions on an organismal and cellular level using immunology, light and electron microscopy.

As we began this project a year and a half ago it became evident that there was a paucity of information regarding the internal morphology of any thrips vector species. Therefore, our first task was to describe the internal morphology of the WFT on an organismal and cellular level, thus creating a "map" of the insect's organs to be used in elucidating the movement and location of TSWV following ingestion of the virus from an infected plant (Ullman, et. al. 1989: Ullman, et. al., in preparation). This basic knowledge of internal morphology has permitted us to gain a preliminary understanding of where TSWV is located within larval and adult WFT following larval acquisition, as well as determining the fate of TSWV in adults feeding on virus for the first time. This paper will focus on what we have

learned about: 1) TSWV location in larval and adult western flower thrips (WFT), Frankliniella occidentalis (Pergande); 2) inter- and intracelluar virus movement; and, 3) why adult thrips cannot inoculate TSWV unless they have acquired virus as larvae.

MATERIALS AND METHODS

INTERNAL MORPHOLOGY. Light, scanning and transmission electron microscopy were used to examine the internal morphology of the WFT, Frankliniella occidentalis. All thrips examined were from colonies maintained on green bean pods in the laboratory.

LIGHT MICROSCOPY. A Wild M8 dissecting microscope (Wild Leitz, Wild Leitz USA, Inc., 1123 Grandview Drive, So. San Francisco, CA 94080) was used to view gross dissections of thrips performed in physiological saline (0.85%). Phase contrast on a Leitz Diaplan compound microscope (Wild Leitz, Wild Leitz USA, Inc., 1123 Grandview Drive, So. San Francisco, CA 94080) was used to further view slide squashes of many of these gross dissections. Several hundred male and female thrips have been dissected and examined in this way. In addition, thick sections of thrips were prepared for viewing with the compound microscope as follows: fixation in 4% glutaraldehyde (in 0.05M cacodylate buffer, pH 7.34) for 4-8 hours, 4 washes in 0.05M cacodylate buffer, post-fixation for 2-4 hours in osmium tetroxide (in 0.05M cacodylate buffer), another 4 washes in 0.05M cacodylate buffer, dehydration in a gradient series of ethanol over a period of 4-6 hours and finally a propylene oxide transition and embedding in Spurrs (Spurr, 1969) (Ted Pella Inc, Redding, CA 96099). After embedding the thrips were serially sectioned at 1 um on a Reichert OMU-2 ultramicrotome in dorso-ventral and sagittal orientations. Sections were mounted on glass slides in a drop of water and heated until sections dried down. In some cases, sections were de-resined in 1.5-3% NaOH (in absolute ethanol). This process was repeated at least 3 times. Then slides were placed in phosphate buffer (pH 4) for 5 min. Slides were further rinsed in double distilled water (DDW) and allowed to air dry prior to staining. A number of stains, including hematoxylin and eosin, geimsa, Mallory's Triple and methylene blue were tried on deresined sections, as well as sections in resin (Humason, 1967). In both cases, we obtained the most consistent results with methylene blue (modified from Humphrey and Pittman, 1974). Fourteen individuals, both male and female were thick sectioned and examined as described.

ELECTRON MICROSCOPY. Thrips were prepared for Scanning Electron Microscopy (SEM) according to the protocol of Hunter and Ullman (1989). After critical point drying, thrips were placed on SEM stubs with silver conductive paint (Sigmund-Cohn Hed Wire, Inc., Mt. Vernon, New York). Each specimen was then coated with silver conductive paint, thoroughly dried and cut longitudinally by lightly dragging a teflon razor blade across either the dorsal or ventral surface of the insect. The cuticle was then carefully pealed back with fine forceps to expose internal structures. These specimens were sputter coated (Hummer II, Technics, 5510 Vine Street, Alexandria, Virginia 22310) and examined with a Cambridge Stereoscan 150, SEM (Cambridge Instruments, UK) at 20 kV. Thirty-two specimens were dissected, viewed and photographed using this technique.

Thrips were prepared for Transmission Electron Microscopy (TEM) by cutting live thrips in half with a teflon razor blade either between the head and thorax or between the thorax and abdomen in a drop of cold physiological saline (0.85%) on a glass microscope slide. A few drops of cold 4% glutaraldehyde (in 0.05M cacodylate buffer, pH 7.34) were then added to the dissection. Immediately thereafter, 2-3 drops of 2% agar (Bacto-agar in 0.05M cacodylate buffer, 40o C) was layered on top of the dissection. After the agar set, a small section containing the

dissected specimen was excised and fixed for 2-4 hours in a vial of 4% glutaraldehyde (in 0.05M cacodylate buffer, pH 7.34). Tissues were processed as described earlier for light microscopy. The embedded specimens were then serially sectioned on a Porter Blum MT-2 microtome, 60-80 nm in thickness and picked up on formvar coated slot grids. Grids were contrasted with 2% uranyl acetate in 50% ethanol for 30-40 min and post-stained with lead citrate in NaOH for 10-15 min. Sections were examined and photographed using a Zeiss 10A transmission electron microscope (Carl Zeiss, West Germany).

VIRUS ACQUISITION AND IMMUNOLOGY. Insects of regulated age from thrips colonies were fed on healthy control plants, sucrose feeding sachets and TSWV infected plants for varying times ranging from 1 h to 4 days. From each group some insects were prepared for viewing with transmission electron microscopy as described herein and some were tested for TSWV presence with enzyme-linked immunosorbent assay (ELISA) using the methods of Cho, et. al. (this proceedings). Methods used for thrips rearing and placements are described by Mau et. al. (this proceedings). Although more than 100 thrips have been tested as described, this work is preliminary and replications are in progress.

Distribution of TSWV in leaves of several host plants was observed using direct tissue blotting on nitrocellulose using a slight modification of the methods of Knecht and Dimond (1984) and Cassaba and Varner (1987).

FEEDING BEHAVIOR. Thrips feeding was observed on sucrose feeding sachets and lettuce using the methods of Hunter and Ullman (1989). Electronic monitoring of feeding behavior was done using an AC electronic monitoring system (Hunter, et al. In Press).

RESULTS AND DISCUSSION

WHY CAN'T ALL THRIPS SPECIES ACT AS VECTORS OF TSWV? WHY MUST VECTOR SPECIES ACQUIRE THE VIRUS IN THEIR LARVAL INSTARS? We hypothesized that the answers to these questions would be complex and involve **behavioral, morphological and physiological** factors as well as specific interactions between the insect, the plant and the virus. The data and interpretations presented herein represent the rudiments of our ongoing efforts to answer these questions and should, for the most part, be considered preliminary. In the following discussion, our data are discussed from the perspective of potential behavioral, morphological and physiological mechanisms influencing vector specificity and acquisition efficiency.

THRIPS FEEDING BEHAVIOR AND VIRUS DISTRIBUTION IN LEAVES. Clearly, if an insect does not feed on a virus source it will be unable to acquire the virus and further act as a vector. Certainly, much vector specificity is a function of whether or not the plant host ranges of the vector and virus overlap. As only larval thrips can acquire TSWV, the reproductive host range of the thrips as it overlaps the TSWV plant host range is most critical. The plant host range of TSWV is noted for its breadth and many thrips species, vectors and nonvectors have overlapping host ranges. Yet, not all the thrips species that feed and reproduce on infected plant hosts act as vectors. One of the first questions we asked was, do these thrips species even ingest virus when they feed from infected plants? Of those species that do act as vectors, do both larvae and adults ingest virus from infected plants? Preliminary ELISA data demonstrates that many species that do not act as vectors of TSWV do indeed ingest the virus during feeding from infected plants (Cho, et. al., these proceedings). Furthermore, both larval and adult WFT ingest virus from infected plants, although the virus titre acquired varies depending upon the plant host species fed upon (Cho, et.

al., these proceedings). These data also show that, based on ELISA readings, the quantity of virus ingested increases as the feeding period increases. It is likely that this phenomenon has much to do with increases in acquisition efficiency following increased feeding on infected plants as noted by Sakimura (1962).

We have demonstrated that WFT feeds in a piercing-sucking manner, emptying individual cells at varying depths within leaf tissue and have described the mouthcone and mouthpart movements during feeding in detail (Hunter and Ullman 1989; Ullman et al. 1989). Electronic monitoring of WFT feeding strongly suggests two modes of feeding: 1) short duration ingestion during which stylets are inserted shallowly, a cell or group of cells are emptied during a few minutes of ingestion, stylets are withdrawn and the process is repeated, and, 2) long duration ingestion during which the stylets are inserted more deeply and ingestion is continuous over 30 minutes or longer (Hunter, et al., In Press). The relationship between different modes of thrips feeding and acquisition and inoculation of TSWV is not yet known; however, it warrants considerable attention as it may be very relevant in directing efforts to breed resistant plants.

Our observations of WFT feeding on various plant hosts, such as cheeseweed, Malva parviflora L., red pualele, Emilia sonchifolia (L.) D.C. and burdock or gobo, Arctium lappa L. suggests that the distribution of WFT feeding on these leaf surfaces varies greatly. Interestingly, preliminary evidence from direct blotting of leaves on nitrocellulose suggests that virus distribution in these plant hosts also varies between species. ELISA results from individual thrips fed on the above plant hosts during larval instars suggests that the amount of virus acquired varies greatly between plant hosts (Cho, et. al., these proceedings; Mau, et. al., these proceedings). One possible explanation is that thrips are behaviorally isolated from the virus on certain plant hosts. Collectively, our data lead one to conclude that the role of thrips feeding behavior and within plant virus distribution on efficiency of virus acquisition and inoculation is important and should be considered in more detail. It is likely that these factors have much to do with variations in efficiency of acquisition and inoculation by thrips.

MORPHOLOGICAL FACTORS. The internal anatomy and morphology of the adult WFT is described and compared to other species that have been studied in Ullman et al (1989). The internal anatomy of the larval instars is similar to the adult, although the looping of midgut is slightly different. We have continued to study the WFT and have noted a large fat body present in male and female larvae and adults. The fat body changes size and shape during insect development. Even when it is somewhat depleted it is quite large extending from the circumoesophageal passage in the brain to the hindgut of the insect. Throughout its length the fat body is intertwined and pressed against the salivary glands, midgut, hindgut and ovarioles. Whole squashes and thin sections of the fat body reveal that it contains large quantities of lipid bodies, sheet-like membranes, golgi bodies and many large vacuoles. The tissue is apparently very metabolically active with many mitochondria and endoplasmic reticulum (Ullman, et al, in preparation) (Fig. 1).

On the level of gross anatomy and morphology, we find no significant morphological differences between larval and adult WFT that might account for the fact that only larval thrips can acquire TSWV. The morphological data we have developed has been tremendously useful to us as they represent our map to the cells and the organs of the WFT and enabled us to conduct the work I will describe in the following paragraphs.

A COMPARISON OF TSWV LOCATION IN WFT FED ON INFECTED PLANTS

AS LARVAE AND AS ADULTS. Using our knowlege of internal morphology, we have developed a technique for dissecting thrips such that the salivary gland stays within the head end of the insect and the oesophagus and gut stays with the tail or abdomen end of the insect. By dissecting in a slightly different manner we can cause the salivary gland to remain attached to the gut and the tail end leaving the head with only feeding structures and neural tissue.

Using ELISA, the intestinal tracts of larvae and adults fed for varying times on TSWV infected plants were positive for the virus. Within one hour virus could be detected in the head end of larval WFT, while heads of adults were not positive, even after 24 hours of feeding of TSWV infected plants (Fig. 2). ELISA readings from larval heads increased as ingestion time increased suggesting continuous movement of virus from the gut to the head region (Cho et. al., this proceedings). As a piece of the fat body described above often remains with the head of the insect, these results may indicate movement to the salivary gland or the fat body. It is very unlikely that we are detecting virus just in the hemolymph as the body parts are bathed in saline prior to placing them in extraction buffer. Furthermore, larval heads devoid of salivary glands were included as a control and these have never tested ELISA positive.

Thin sections of adult WFT from the above cohorts revealed virus in the midgut lumen and midgut epithelial cells of insects fed on infected plants (Fig. 3). Virus was not seen in control WFT fed on healthy plants. In these adults, feeding for the first time on an infected plant, the virus apparently moves from the gut lumen into the epithelial cell by endocytosis (Fig. 3) and intracellular transport via coated vesicles appears to occur. Virus within the epethelial cell lumen appears large groups encircled by rough endoplasmic reticulum (RER) (Fig. 4). These finding suggest that receptor-mediated endocytosis is occuring and very specific receptors exist on the virus and the apical plasmalemma of the epithelial cell and coated vesicles within the cells (Goldstein, et. al. 1985; Simons and Fuller 1985). Receptor-mediated movement between cells would serve to explain much vector specificity. To-date we have not seen virus in the fat body or salivary gland of adult WFT fed for the first time on TSWV infected plants.

In contrast, we do find virus in the gut lumen, midgut epithelial cells and fat body of larval thrips feeding for the first time on TSWV infected plants (Fig. 5). As we have not completed thin sectioning of the insects for TEM viewing, we cannot as yet say if TSWV is present in the salivary gland although we expect to find it there as well. These results suggest that perhaps the inability of adult thrips to inoculate TSWV unless acquisition occurs as larvae is due to impermeability of the midgut basement membrane. Encircling of the virus by RER in adult epithelial cells and ELISA data showing the decrease in virus presence over time following acquisition by adult WFT (Cho, et. al., these proceedings) suggest that TSWV is also sequestered and destroyed within these cells. Our observations using TEM and immunological data strongly suggest that WFT midgut epithelial cells are the end fate of TSWV in adult thrips that did not acquire virus as larvae. These hypotheses bear further investigation and represent a portion of our current and future research plans.

The fat body we have described herein apparently plays an important role in WFT/TSWV interactions. TSWV is present in larval fat body and in fat body of adults reared from larvae fed on TSWV infected plants. The virus appears to be in membrane bound vesicles and in thin sections there is a great deal more virus in approximately 7 day old adults that acquired TSWV as larvae. The fat body is greatly diminished during pupation as

the pupal instars do not feed. It is interesting to note that ELISA data indicates that TSWV virtually dissapears during pupation (Cho, et. al., these proceedings). Perhaps the fat body is a primary site for transtadial passage.

DOES TSWV REPLICATE IN THRIPS CELLS? We have developed several lines of evidence that strongly suggest that TSWV does replicate in thrips cells. ELISA data shows that while the virus is transtadially passed, it nearly dissapears during pupation and increases over time during adulthood (Cho, et. al., Mau, et. al., these proceedings). We have seen crystalline arrays, similar to those present in tissues where viruses are replicating, in the fat body of larval thrips that have fed on TSWV for several days (Figs. 1 & 6). These crystalline arrays have been seen only in insects from cohorts that test ELISA positive for TSWV. They have never been seen in cohorts of insects fed on healthy plants and testing ELISA negative. While we do not have direct evidence that these crystalline arrays are associated with TSWV or that TSWV is replicating in the fat body, these data are suggestive. Furthermore, there appears to be more virus in 7-10 day old adults that acquired virus as larvae than in larvae from the same cohort just following acquisition. In collaborative work with Dr. T.L. German, we are currently feeding WFT on TSWV infected or healthy plants and dissecting out individual salivary glands, fat bodies and midguts for testing with TSWV strand specific probes (German, et. al., this proceedings). Our future plans include doing in situ hybridizations of these tissues to provide direct evidence for or against replication of TSWV in various thrips cells.

SUMMARY AND CONCLUSIONS. In light of the collaborative work presented herein we hypothesize that the primary mechanisms governing specificity and acquisition by larval and not adult thrips are largely physiological, involving membrane permeability receptor-mediated endocytosis. Our results are quite preliminary and great deal of work remains to be done before we can truly define the specific physiological mechanisms operating in this system. The role of feeding behavior and virus distribution within different plant hosts warrants further consideration, particularly as they relate to acquisition and inoculation efficiency. Finally, plant host range and feeding behavior play an important role in limiting the number of species coming in contact with TSWV and remain very important in developing strategies to limit the spread of TSWV.

LITERATURE CITED

Cassaba, G.I. and J.E. Varner. 1987. Immunocytolocalization of extensin in developing soybean seed coats by immunogold-silver staining and by tissue printing on nitrocellulose paper. Cell Biology 105:2581-2588.

Goldstein, J.L., Brown, M.S., Anderson, R.G.W., Russel, D.W. and J.S. Wolfgang. 1985. Receptor-mediated endocytosis: Concepts emerging from the LDL receptor system, Ann. Rev. Cell Biol. 1:1-39.

Hunter, W.B. and D.E. Ullman. 1989. Analysis of mouthpart movements during feeding of Frankliniella occidentalis (Pergande) and F. schultzei Trybom (Thysanoptera: Thripidae), Int. J. Insect Morphol. & Embryol. 18:161-171.

Hunter, W.B., Ullman, D.E. and A. Moore. Electronic monitoring of thrips feeding using an AC system and computer interface. Misc. Publications of the ESA (In Press).

Knecht, D.A. and R.L. Dimond. 1984. Visualization of antigenic protiens on western blots, Analytical Biochem. 136:180-184.

Sakimura, K. 1962. The present status of thrips-borne viruses. pp. 1-192. In K. Maramorosch (ed.) Biological Transmission of Disease Agents. Academic Press, New York.

Simons, K. and S.D. Fuller. 1985. Cell surface polarity in epithelia, Ann. Rev. Cell Biol. 1:243-88.

Ullman, D.E., Westcot, D.M., Hunter, W.B. and R.F.L. Mau. 1989. Internal anatomy and morphology of Frankliniella occidentalis (Pergande) (Thysanoptera: Thripidae) with special reference to interactions between thrips and tomato spotted wilt virus, Int. J. Insect Morphol. & Embryol. 18:289-310.

Ullman, D.E., Westcot, D.M., Cho, J.J. and R.F.L. Mau. Tomato spotted wilt virus in cells of the western flower thrips, Frankliniella occidentalis (Pergande) (Thysanoptera: Thripidae) (In Preparation).

Figure 1. Transmission electron micrograph of the fat body (FB) in a larval western flower thrips where it presses against the brain (B). Note the sheet like layers of membrane, numerous vacuoules and mitochondria. The arrow denotes a crystalline array shown at higher magnification in Fig. 6.

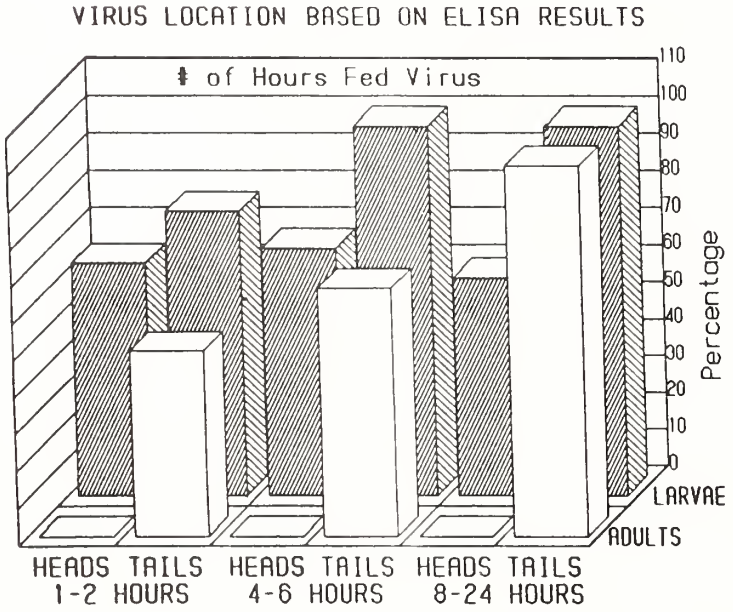

Figure 2. Percent tomato spotted wilt virus positive larval and adult heads and tails based on testing with enzyme-linked immunosorbent assay.

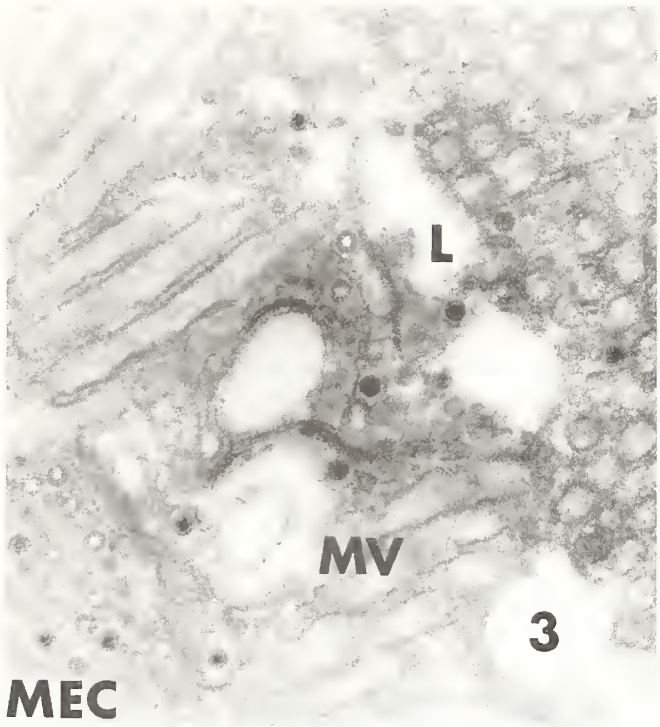

Figure 3. Tomato spotted wilt virus particles within the midgut lumen (L), midgut microvilli (MV) and the lumen of the midgut epithelial cell (MEC).

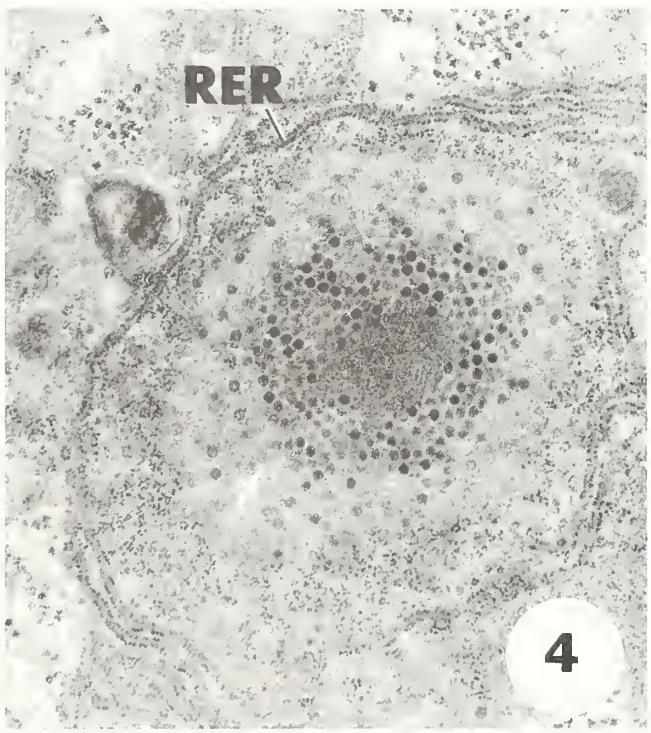

Figure 4. Tomato spotted wilt virus particles (denoted by arrow) encircled by rough endoplasmic reticulum (RER) within the midgut epithelial cell of an adult western flower thrips.

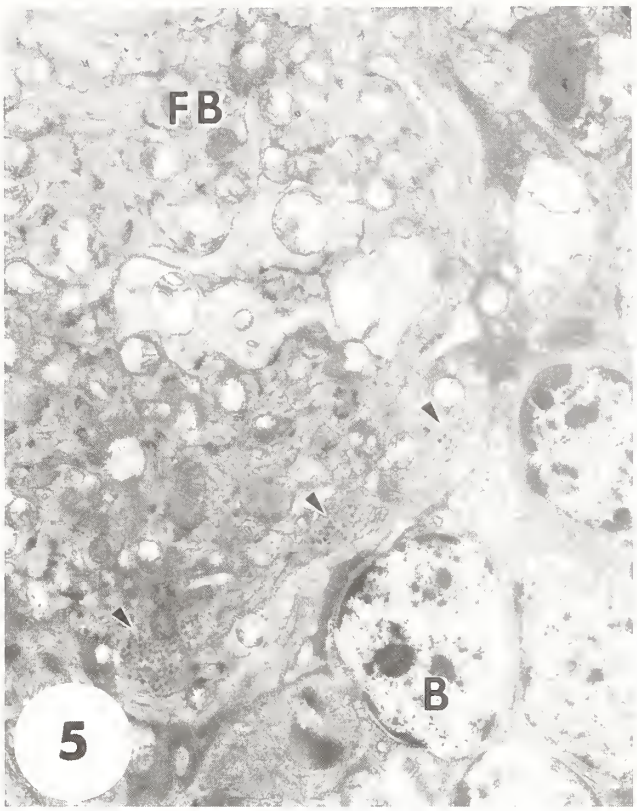

Figure 5. Tomato spotted wilt virus particles in membrane bound vesicles (denoted by arrows) in the fat body (FB) of a larval western flower thrips.

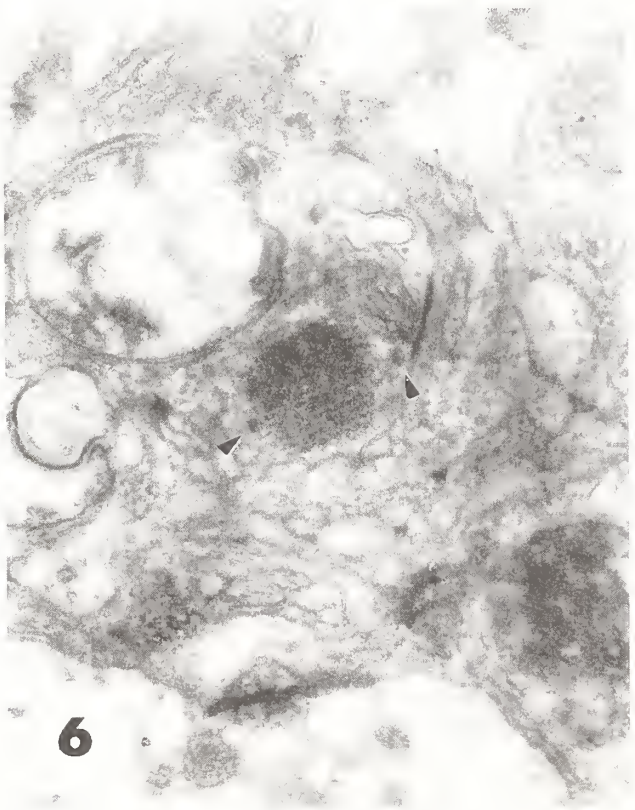

Figure 6. A crystalline array within the fat body of a larval western flower thrips. Virus particles are denoted by arrows.

DETECTION OF TOMATO SPOTTED WILT VIRUS RNA IN PLANTS AND THRIPS USING STRAND-SPECIFIC PROBES

T. L. German, Yi Hu, Ron Mau and Diane Ullman

ABSTRACT

A diagnostic dot blot procedure for tomato spotted wilt virus (TSWV) was developed using cDNA clones. This assay is useful for detecting the presence of virus in all hosts tested and in individual thrips (Rice et al, 1990). One of these clones (pTSWV80) was subcloned into the vector Blue Script II to take advantage of the T7 and T3 promoters for the purpose of generating strand-specific probes. Probes were generated and used to determine the time course of appearance of each strand in plants and to demonstrate the presence of both strands in thrips. Although we have not assigned polarity with respect to translation of the strands, these data indicate that both (+) and (-) sense RNAs occur in insect vectors of TSWV.

INTRODUCTION

A cDNA library was constructed that contains sequences complementary to tomato spotted wilt virus (TSWV) RNA. Two of these plasmids, pTSWV7 and pTSWV80, containing inserts of 460 and 870 base pairs were characterized for specificity to TSWV RNA and selected to develop a dot blot assay. ^{32}P labeled hybridization probes can detect TSWV in as little as 16 ng of total RNA from tobacco, 80 ng from tomato and lettuce and 400 ng in chrysanthemum and pepper. TSWV is detected by these hybridization probes in dot blots of nucleic acids extracted by simple methods using 40-50 mg of leaf tissue (Figure 1). These results show that the probes have complete specificity for TSWV infected plant material and that the probes will detect viral RNA in pooled thrips which have been reared on infected plant material. Since five thrips were pooled in the least dilute sample on this blot and five-fold serial dilutions were performed, the assay appeared sensitive enough to detect viral RNA in less than an individual insect.

To further examine the utility of this assay for detecting viral RNA in insects, an experiment was done with groups of larval, prepupal and adult thrips that were allowed access to either healthy or TSWV infected plants and then were blotted to nitrocellulose filters and probed. The results in Figure 2 show that some insects from each developmental stage fed on TSWV infected plants tested positive for virus while all thrips fed on healthy control plants tested negative. As expected, individual thrips exhibited considerable variability in the amount of signal which they generated in the assay and some insects from each developmental stages tested positive. These results suggested that we might be able to use strand-specific probes to address the question of whether or not TSWV replicates within thrips. We reasoned that whatever the sense of virion RNA with respect to translation, there should be only one polarity of each strand incapsidated in virus particles and both strands would be present in any tissues in which virus replication was occurring. Our approach was to make strand specific probes and verify that only one strand would hybridize to virion RNA, but that both strands would hybridize to plant tissue in which replication is known to occur and then use these probes to determine whether one or both viral strands are present in the tissues of thrips that have fed on infected plant material.

MATERIALS AND METHODS

The viral strain description, growth and purification, synthesis and cloning of TSWV specific cDNA and Frankliniella occidentals (Pergande) manipulations were previously described in detail (Rice et al, 1990). Only relevant aspects of those procedures will be described here.

Tissue for leaf disc blot assays (Figure 1) was extracted from 40-50 mg of material by homogenizing in 125 ul of extraction buffer (Owens and Diener, 1981), in a 1.6 ml microfuge tube using a plastic tight fitting pestle. 125

ul of buffer saturated phenol was added and the tubes were vortexed. 125 ul of chloroform:isoamyl alcohol (24:1) was added and the suspension was vortexed again. The phases were separated by microcentrifugation for 1 min and 75 ul of the clear upper phase was transferred to a clean Eppendorph tube containing 175 ul of 6.15 M formaldehyde in 10 X SSC (1 X SSC = 0.15 M NaCl, 0.015 M NaCitrate pH 7.0). Serial dilutions were made in the same buffer. The samples were heated to 65 C for 15 min and blotted onto nitrocellulose.

RNA was isolated from thrips by the acid guanidinium thiocyanate-phenol-chloroform (AGPC) extraction method as described by Chomczynski and Sacchi (1987). The insects were frozen in liquid nitrogen and homogenized in a microfuge tube with 0.1 ml of 4.2 M guanidinium thiocyanate, 0.025 M sodium citrate pH = 7.0, 0.5% sodium sarcosyl and 0.01 M 2-mercaptoethanol. 50 ul of this was transferred to a microfuge tube containing 150 ul of 6.15 M formaldehyde in 10 X SSC and heated to 65 C just prior to blotting.

General probes (not strand specific) for dot blots were prepared as follows. Plasmids pTSWV7 and pTSWV80 were purified by the Sadhu and Gedamu (1988) procedure, inserts were released by digestion with Pst I (Maniatis, 1982), and separated from plasmid bands by electrophoresis in 4% NuSeieve GTG agarose (FMC). The gel was stained with 0.2 ug/ml of ethidium bromide in water and desired band was excised and radiolabeled with ^{32}P in the melted gel slice using the random primer extension technique of Feinberg and Vogelstein (1983, 1984).

Subcloning was carried out as follows. A Pst I fragment was isolated from pTSWV80 as described above, purified by phenol extraction and ethanol precipitation. The isolated fragment was ligated into the Pst I site of dephosphorylated plasmid vector Blue Script II as described by Maniatis et al (1982). Competent E. coli strain XL1-Blue cells were transformed and plated on LB media containing 100 ug/ml ampicillin, IPTG and X-Gal. White colonies were isolated, plasmids were purified from large scale preparations and analyzed for the presence of TSWV specific inserts. Restriction analysis was done to insure that only one insert was present in the selected subclone. Restriction analysis was also used to determine that EcoR I and Sph I would produce linear molecules that could be used for run off synthesis of nucleic acid complimentary to both strands of the subcloned insert by utilizing the T7 or T3 specific promoters which flank the polylinker region of the Blue Script II vector.

To synthesize strand-specific probes, the primer extension procedure of Schuler and Zielinski, 1989, was used. Plasmids were linearized with EcoR I or Sph I and a subsample was run on a gel to insure that it had been completely digested. 1.5 ug of linearized plasmid DNA in 8.0 ul of TE buffer was added to 2 ul of 2 N NaOH and 2 ul of EDTA, mixed and incubated at room temperature. The denatured DNA was ethanol precipitated, collected by centrifugation and washed with 70% ethanol. The dried pellets were re-dissolved in 6 ul of TE, 1 ul of 10 X DNA polymerase buffer (100 mM Tris-Cl pH 7.5, 100 mM $MgCl_2$, 500 mM NaCl and 50 mM dithiothreitol), 4 ul of a 2.4 mM solution of dATP, dTTP, dGTP and 0.24 mM dCTP, 30 ng of a 18 bp primer specific for either the T3 or T7 promoter, 10 ul of a ^{32}P dCTP (10 uCi/ul, 400 Ci/mmole) and 4 ul of Klenow fragment (1 unit per ul). This reaction mixture was incubated for 1 hr at 37 C, ethanol precipitated and used as a hybridization probe without further treatment.

Blotting and hybridization protocols were selected from Wahl et al, 1987. Samples were applied to nitrocellulose membranes with vacuum dot blot apparatus (Bio-Rad). Prehybridization solution was 50% (v/v) formamide, 5 X SSPE, 5 X Denhardt's, 0.2% SDS, 200 ug/ml yeast t-RNA and 200 ug/ml low molecular weight salmon sperm DNA. Hybridization was done in prehybridization buffer containing probes (10^6 cpm/ml) at 42 C for about 18 hrs. Hybridized blots were washed with a final stringency of 0.1 X SSPE/0.2% SDA at 60 C for 1 hr. Membranes were exposed to Kodak X-Omat AR film at -80 C with intensifying screens for various time periods.

RESULTS AND DISCUSSION

Blots prepared with replicates of infected plant material from 0 to 16 days post-inoculation (dpi) and hybridized with the probe prepared with either the T3 or T7 primer product are shown in Figure 3. The T3 primer generates a probe that will detect a small amount of viral RNA over the course of the infection starting from about 4 dpi. Note that the concentration of this strand is such that it is detected only at the lowest dilution applied to the paper at any given time point. By contrast, the T7 directed primer detects viral RNA with greater intensity and is able to produce a signal at several dilutions over the range of days from about 2 dpi to 16 dpi. The signal on the lower right of these blots (1d-4d) is from equal amounts of plasmid DNA containing the insert from which the probes were generated showing that the difference in intensity of the signal observed with the two different probes is not due to differences in the specific activity of the probe but rather to a difference in the amount of target RNA in the tissue samples.

The left side of Figure 3 shows the signal obtained when the two probes are hybridized to a blot of purified virus (VR). Again, the plasmid controls (1d-3d) show that the signal difference is not due to probe specific activity. The RNA in the spot from infected plant RNA (PR) gives the same type of signal with the two probes as the time course experiment on the right. Significantly, the viral RNA source gives a very strong signal with the T7 primer generated probe and no signal with its complimentary sequence generated from the T3 primer probe. These data prove that the T7 probe is the compliment of the viral RNA strand and that the T3 probe does not cross-hybridize to it.

The results of a similar experiment using RNA isolated from thrips is shown in Figure 4. The right side of this figure is the same as that in Figure 3 and is shown for reference purposes. As in Figure 3, PR is an RNA sample from plants which are 16 dpi and spots 1d-2d are linearized vector identical to that used to transcribe the probes used in these experiments. The spots marked T1, T2 and T3 contain, respectively, RNA isolated from larval thrips fed on infected plants for 2 days, RNA from adult thrips fed on infected plants for 2 days and thrips fed on healthy plants for 2 days. As shown by the strong signal from the T7 probe, the larval thrips fed on infected plants acquired and retained virus. The weak signal produced by the RNA from these same larvae tested with the T3 generated probes suggests that the RNA strand complimentary to the packaged strand is present. Interestingly, the adult thrips (spots T2) did not give a signal with either probe. This may have occurred because none of the insects in this particular sample acquired virus or the virus may have passed through the adults before the RNA was extracted. The absence of signal from the strand that occurs in lower concentration in the adults might indicate that replication only occurs in larvae.

An interpretation of these data is that the T7 probe is detecting the viral RNA strand that is packaged into virions and the T3 probe is detecting its replicative template. The data from plant samples tested over a period of time would substantiate this hypothesis. If, indeed, this is the case, the data from the thrips experiments suggests that replication is occurring in insects as well as plants. The necessity of having to rely only the weak signal from insects makes a definitive conclusion difficult. However, more recent results (data not shown) using RNA probes transcribed from the T7 and T3 promoters with the corresponding RNA polymerases have confirmed the data from the experiments presented here.

REFERENCES

Chomczynski, P. and N. Sacchi. 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162:156-159.

Feinberg, A. P. and B. Vogelstein. 1983. A technique for radiolabeling DNA restriction endonulcease fragments to high specific activity. Anal. Biochem. 132:6-13.

Feinberg, A. P. and B. Vogelstein. 1984. Addendum: A technique for radiolabeling DNA restriction endonuclease fragments to high specific activity. Anal. Biochem. 137:266-267.

Maniatis, T., E. F. Fritsch and J. Sambrook. 1982. Molecular Cloning: A Laboratory Manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Owens, R. A. and T. O. Diener. 1981. Sensitive and rapid diagnosis of potato spindle tuber viroid disease by nucleic acid hybridization. Science 213:670-672.

Rice, D. J., T. L. German, R. Mau and F. Fujimoto. 1990. Dot blot detection of tomato spotted wilt virus RNA in plant and thrips tissues by cDNA clones. Plant Disease 74:274-276.

Sadhu, C. and L. Gedamu. 1988. A procedure for the preparation of RNA-free plasmid DNA. Biotechniques 6:20-21.

Schuler, M. A. and R. E. Zielinski. 1989. Methods in Plant Molecular Biology. p. 136. Academic Press, Inc., San Diego, California.

Wahl, G. M., E. Ong, J. Meinkoth, R. Franco and M. Barinago. 1987. Transfer and immobilization of nucleic acids to Schleicher and Schuell solid supports. pp. 1-34. Schleicher and Schuell Publication #700. Schleicher and Schuell, Inc., Keene, New Hampshire.

T. L. German is an Assistant Professor, Department of Plant Pathology, University of Wisconsin-Madison, Madison, WI 53706; Yi Hu is a Graduate Research Assistant, Department of Microbiology, University of Hawaii, Honolulu, HI 96822; and Ron Mau and Diane Ullman are Professor and Assistant Professor, respectively, Department of Entomology, University of Hawaii, Honolulu, HI 96822.

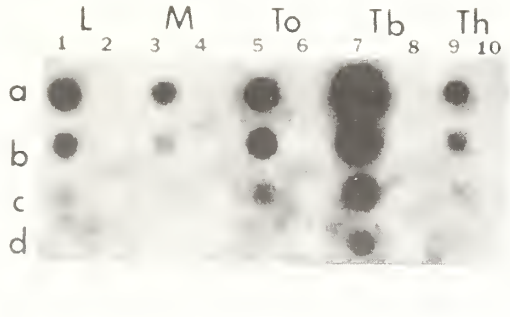

FIGURE 1.

Dot blot hybridization starting with equal masses (40-50 mg) of plant leaf disc tissue or 5 thrips. Rows a-d are five-fold serial dilutions of starting material. Letters over columns represent lettuce (L), chrysanthemum (M), tomato (To), tobacco (Tb), and thrips (Th). Infected samples are odd numbered lanes, and uninfected samples are even numbered lanes.

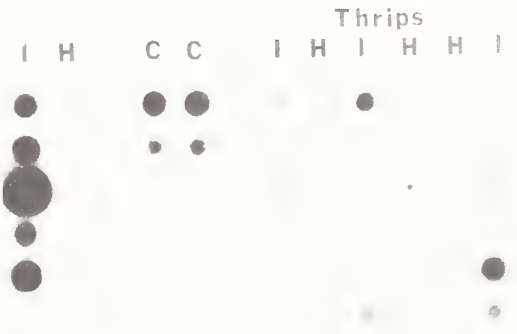

FIGURE 2.

RNA dot blot detecting individual TSWV infected thrips at three major developmental stages in the life cycle. Larval thrips were fed on TSWV infected (I) burdock leaves or healthy (H) bean pods and harvested at adult, pre-pupal, and second instar larval stages of development. RNA in infected and healthy pair columns (from left to right): individual second instar larvae, individual pre-pupal, individual adults.

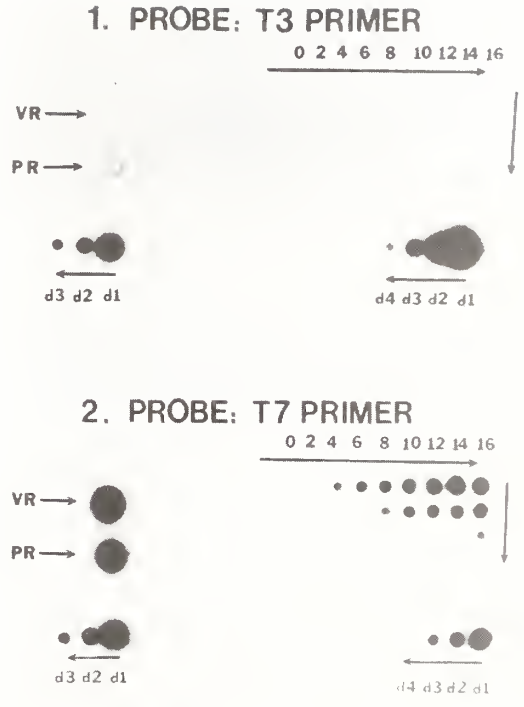

FIGURE 3.

Right side of each panel - Hybridization of strand specific probes (T3 or T7) to plant tissue harvested from 0 to 16 days post-inoculation. Numbers at the top of the figure are days past inoculation. The arrow down the right side indicates the dilution series. Spots labeled 1d-4d are a dilution series of the plasmid used to construct the strand specific probes starting with 44 ng.

Left side of each panel - Strand specific probes hybridized to plant RNA from a 16-day-old infection (PR) and to purified virus (VR). The 1d-3d is control plasmid DNA in a dilution series.

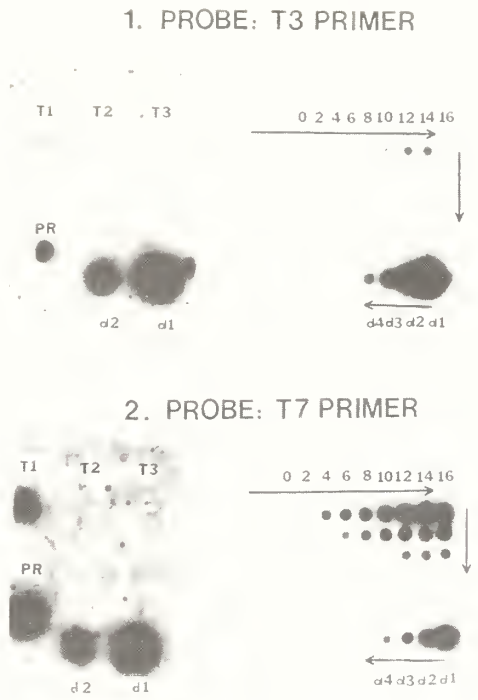

FIGURE 4.

Right side of each panel - Same as Figure 3 with a longer exposure.

Left side of each panel - Hybridization of strand specific probes to RNA from plants 16 dpi and to larval thrips fed for 2 days on infected plants (T1), adult thrips fed for 2 days on infected pants (T2) and thrips from uninfected plants (T3).

DETECTION OF THE TOMATO SPOTTED WILT VIRUS (TSWV) WITHIN THRIPS

J. J. Cho, R. F. L. Mau, D. E. Ullman, and D. M. Custer

ABSTRACT

Detection of TSWV in individual thrips has been used to address fundamental questions regarding virus-vector relationships. Thrips ELISA has been used for routine determinations to assess suitability of acquisition plant materials and acquired virus titers. TSWV was passed transstadially in Frankliniella occidentalis through several molts and pupation when allowed acquisition access as larvae. Trans-stadial passage was not observed in Hercinothrips femoralis, a non-vector thrips species. Virus replication within F. occidentalis was suggested in two tests based on high titers detected in late stage adults. Percentage of virus positive individuals and virus titers rapidly declined in F. occidentalis after ingestion as adults. TSWV was detected in head-thorax and abdomen parts of F. occidentalis allowed acquisition access as larvae.

INTRODUCTION

The tomato spotted wilt virus (TSWV) is the only known plant virus to be transmitted in a circulative manner by certain species of thrips. Pittman in 1927 was first to establish that onion thrips, Thrips tabaci Lindeman, transmitted TSWV in tomato. Thus far, only five other thrips species including: Frankliniella fusca (Hinds), F. occidentalis (Pergande), F. schultzei (Trybom), Scirtothrips dorsalis (Hood), and T. setosus Moulton have been established as vectors of TSWV (Amin, 1981; Gardner et al, 1935; Kobatake, 1984; Sakimura, 1963; Samuel et al, 1930).

Previous work established that TSWV can only be acquired by larval stages of thrips after a minimum feeding period of a few minutes. However, transmission efficiency increases with concomitant increase in the acquisition period. A latent (incubation) period is necessary before transmission can occur (Bald and Samuel, 1931). The duration of the latent period varies with different thrips species. Larval thrips are able to transmit the virus before they pupate, but adults more commonly transmit the virus (Sakimura, 1962). Adults can remain infective
throughout their life, however, virus transmission is erratic.

Although thrips have been recognized as TSWV vectors for more than sixty years, the virus-vector interaction is still not fully understood. Questions relating to acquisition, persistence, replication, and location of TSWV within thrips and how these factors are related to effective transmission still remains unanswered. Specifically, acquisition data are limited, the latent period is variable between species, retention is erratic or inconsistent and appears to be related to the amount of virus acquired upon acquisition, transmission is erratic and efficiency maybe variable, the virus is persistent in vector thrips but little is known how it survives or whether it propagates within the insect, several thrips species have been demonstrated to be non-TSWV vectors but the mechanisms for this phenomenon is unknown.

Previously, we reported the use of the enzyme-linked immunosorbent assay (ELISA) for the detection of TSWV in individual thrips (Cho et al, 1988). In that study, we were able to readily detect TSWV in individual thrips previously allowed access on TSWV infected plants in the laboratory and from the field. In this paper we will discuss the utility of ELISA

Cho is a professor in the Department of Plant Pathology, University of Hawaii, CTAHR - Maui County Research, Kula, HI 96790; Mau is an extension specialist and professor in the Department of Entomology, University of Hawaii, Honolulu, HI 96822; Ullman is an assistant professor in the Department of Entomology, University of Hawaii, Honolulu, HI 96822; Custer is a research associate in the Department of Plant Pathology, University of Hawaii, CTAHR - Maui County Research, Kula, HI 96790.

for addressing many of the unanswered fundamental questions relating to TSWV-vector relationships. For example, we have used this method routinely to determine acquisition by larval thrips, the fate of TSWV in vector vs non-vector thrips, virus persistence in vector thrips ie. transtadial passage and adult retention, virus multiplication in thrips, and vector potential in the field.

MATERIALS AND METHODS

Enzyme-Linked Immunosorbent Assay

The double antibody sandwich ELISA method was used to detect TSWV in individual thrips. A polyclonal antiserum produced from a TSWV strain obtained from an infected lettuce plant on the island of Maui (Gonsalves and Trujillo, 1986) was kindly provided by D. Gonsalves (Cornell University, Geneva). We have used this polyclonal antiserum extensively to detect TSWV using ELISA in reservoir weed hosts of TSWV in Hawaii's vegetable growing regions, and to confirm TSWV infection of inoculated greenhouse-grown plants (Cho et al, 1986). Individual thrips were removed from storage vials with a fine-tipped camel's hair brush, placed into separate wells of a polyvinyl chloride microtiter plate (Dynatech, Alexandria, VA), triturated with the blunt end of a small glass rod and 50 µl ELISA extraction buffer (0.01 M sodium-potassium phosphate buffer, pH 7.4 containing 0.02% sodium azide [w/v], 0.8% sodium chloride [w/v], 0.05% Tween 20 [v/v] and 2% polyvinylpyrrolidone, mol wt 40,000 ([w/v] Sigma Chemical Co., St. Louis, MO) added per well. The suspension was transferred to U-bottom Immulon 2 microelisa plates (Dynatech, Alexandria, VA) previously coated with 200 µl TSWV immunoglobulin (1 µg/ml) and incubated overnight at 4 C. Five to six wells were filled with ELISA extraction buffer without insect extract. TSWV-infected Nicotiana tabacum L. tissue sap 1/50 [w/v] prepared in ELISA extraction buffer was added to other wells.

Alkaline phosphatase-conjugated immunoglobulins were cross absorbed with healthy N. benthamiana tissue extract (1:20, w/v) for 15 min at room temperature (25 C to 27 C), added at 1/2000 dilution, and incubated for 4 to 5 hr at 30 C. Plates were washed three times between each step with phosphate-buffered saline containing 0.05% Tween 20. p-nitrophenyl phosphate (Sigma Chemical Co., St. Louis, MO) was added at 1 mg/ml, incubated for 1 hr at room temperature. ELISA reactions were measured spectrophotometrically at 405 nm and 490 nm using a dual beam EL309 EIA reader (Bio-Tek Instruments, Winooski, VT). The A405 nm values presented were obtained by subtracting the buffer control absorbance values (average of three wells) from sample values. A sample was considered TSWV-positive if the A405 nm values were greater than the mean healthy thrips control values plus three standard deviations.

Thrips

Insects used in our studies were obtained from laboratory-reared colonies of thrips. Virus-free colonies of two TSWV vector species, F. occidentalis, and F. schultzei (pale form), were maintained on bean pods (Phaseolus vulgaris L. cv. Green Crop) and of a non-vector species, Hercinothrips femoralis, (Sakimura, 1946) were maintained on chrysanthemum leaves in the laboratory. Colonies were started from nymphs which emerged from eggs oviposited on bean pods by F. occidentalis, H. femoralis from adults collected from Chrysanthemum morifolium (Ram.) Hemsl., and F. schultzei from adults collected from Leucaena glauca L. blossoms obtained from the field. Thrips colonies were maintained at 26 C with a 10-hr light and 14-hr dark cycle.

Thrips were placed in small vials containing 50 µl of ELISA extraction buffer and stored at 4 C until analyzed for TSWV by ELISA.

TSWV Acquisition And Retention Studies

Laboratory-reared first instar larvae were either allowed free access or caged on the upper leaf surfaces of virus source plants for acquisition access feeding. Separate groups of first instar thrips were also allowed free access or caged on virus free plants as a control treatment. Source plants were TSWV-infected Emilia sonchifolia (L.) DC, Arctium lappa L., or Datura stramonium L. plants. After acquisition, source plants were removed and vector thrips species were allowed to complete their development on TSWV-free bean pods; H. femoralis completed their development caged on TSWV-free chrysanthemum leaves in modified Tashiro cages (Tanigoshi, 1982). In studies designed to follow the fate of TSWV within thrips, subsamples of the desired stage of thrips development were randomly selected, removed from the developing populations and analyzed by ELISA.

A technique was developed to facilitate location of TSWV within organs of F. occidentalis and aid in selection of possible virus containing individuals for transmission electron microscopy (TEM) work. Larval and adult thrips were allowed access to TSWV infected plants for various times up to 24 hrs. Individual insects were dissected into a head section which also contained the oesophagus, and a tail section or the abdomen which contained the gut and other internal organs.

RESULTS AND DISCUSSION

TSWV Acquisition By Vector Thrips

Several tests have been directed at determining optimal conditions for TSWV acquisition. Our data indicate that the acquisition process is not as simple as just placing thrips on infected source plants and then running a series of transmission studies. With ELISA we were able to demonstrate that the percentage TSWV positive F. occidentalis larval individuals and virus titer between individuals varied with host plant species used as virus sources and the length of the feeding period (Figure 1). A higher percentage of

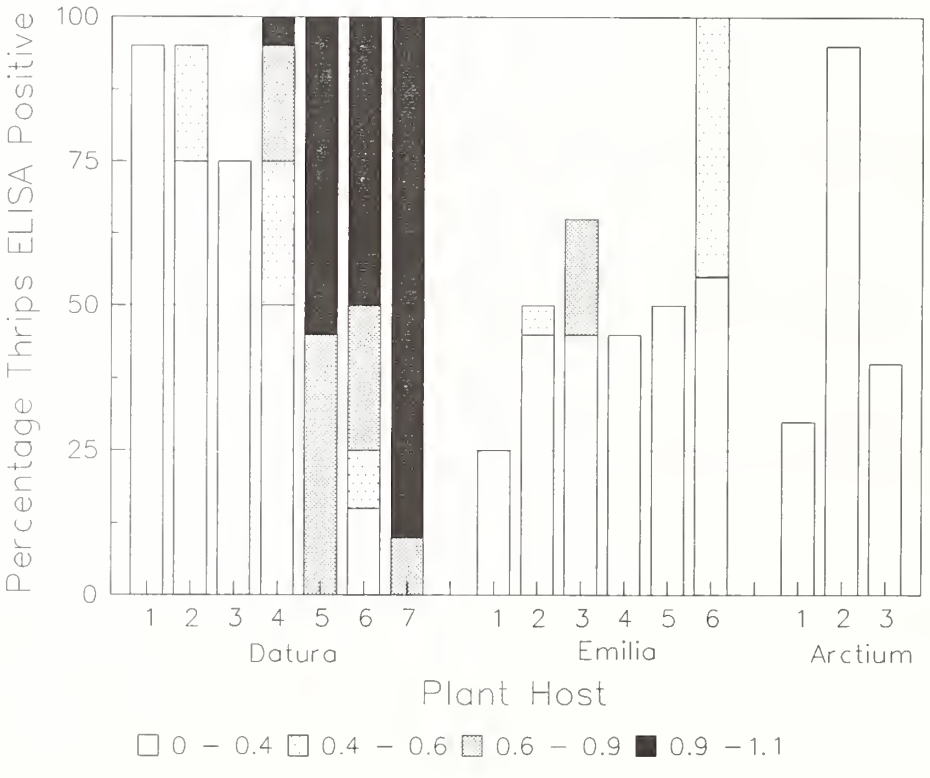

Figure 1. Comparison of percentage of Frankliniella occidentalis larvae testing positive for TSWV by ELISA after acquisition access feeding on TSWV-infected Datura stramonium, Emilia sonchifolia, and Arctium lappa plants.

individuals were ELISA positive and and acquired higher virus titers when fed on D. stramonium as compared with E. sonchifolia, and Arctium lappa. This phenomenon is probably associated with the distribution of TSWV within different host plants.

In our study comparing virus location within head vs tail sections we found that F. occidentalis larvae acquire virus more quickly than adults with 45% of the larvae acquiring after 1 hour compared to only 30% of adults acquiring in the same time (Figure 2). After 24 hrs of feeding access to TSWV-infected plants there was no significant difference in quantity of virus ingested by the two life stages. Furthermore with larvae, virus appears to be quickly transported from the gut into the salivary gland based on detection of TSWV associated with the head sections of individuals (Figure 3).

In contrast, adult thrips given access to TSWV, ingest and retain virus for several days; however, virus is not translocated to the salivary glands. This may explain why only adults that have acquired TSWV as larvae can become inoculative. Virus levels based on A405 nm values appeared to increase with an increase in the acquisition access feeding period (Figure 4). The results from this work are preliminary and a more definitive analysis will be forthcoming.

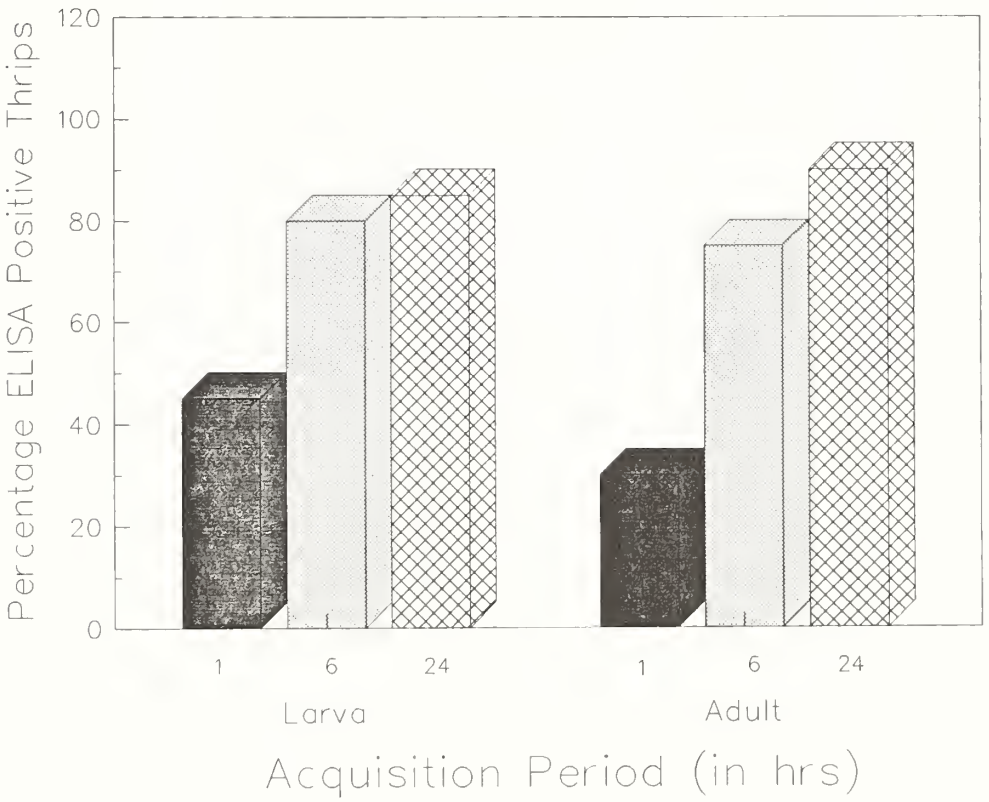

Figure 2. Percentage of Frankliniella occidentalis testing positive for TSWV by ELISA after various acquisition access periods as larvae and adults.

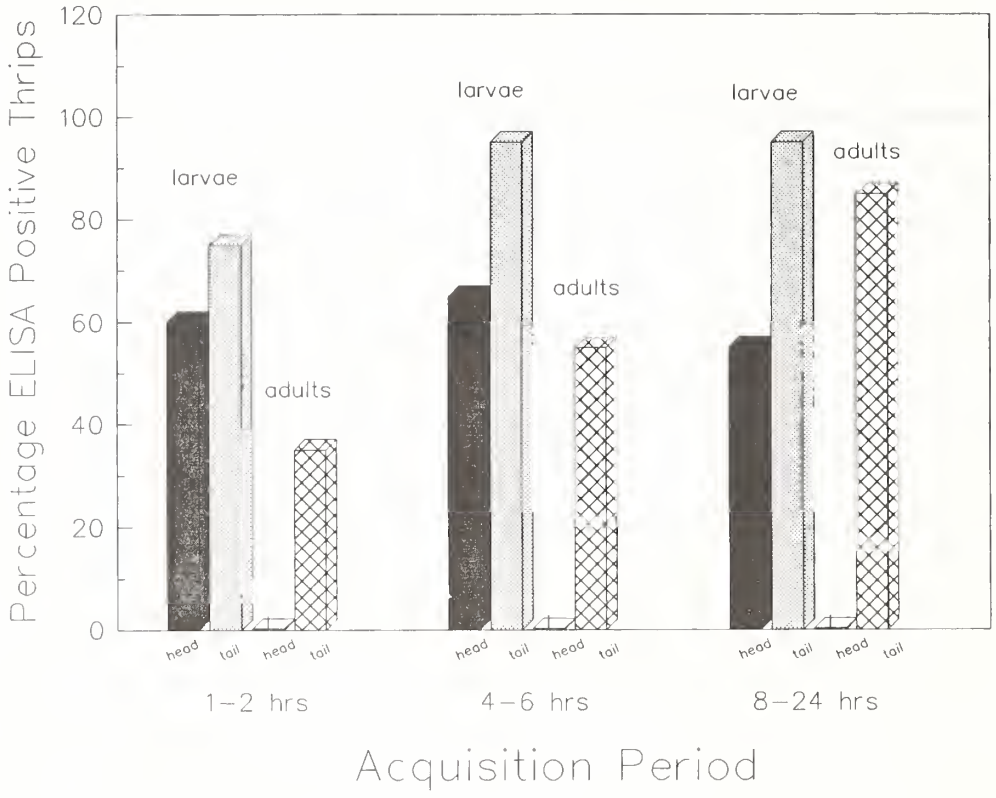

Figure 3. Percentage of the head- and tail-sections of Frankliniella occidentalis testing positive for TSWV by ELISA after various acquisition access periods as larvae and adults.

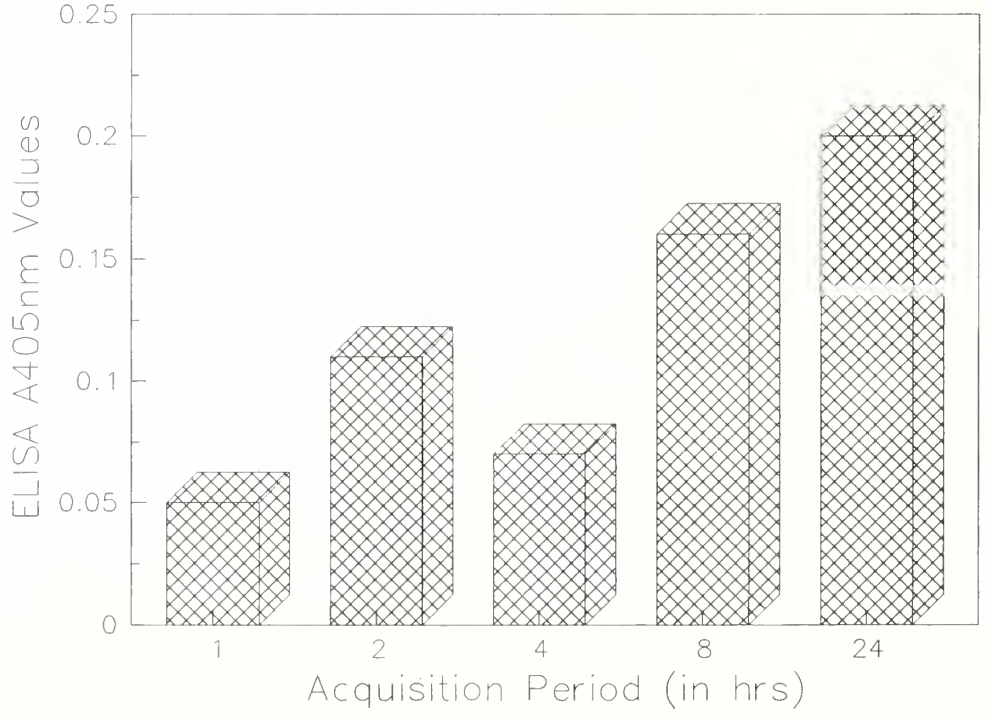

Figure 4. Percentage of larval head-sections of Frankliniella occidentalis testing positive for TSWV by ELISA after various acquisition access periods.

Persistence Of TSWV In Vector And Non-Vector Thrips

Three different experiments were initiated to determine the fate of TSWV in vector and non-vector thrips. Studies compared persistence of TSWV 1) in vector thrips (F. occidentalis) after an access feeding as larvae; 2) in non-vector thrips (H. femoralis) after access feeding as larvae; and 3) in a vector species (F. occidentalis) after access as adults.

When F. occidentalis was allowed access as larvae, TSWV could be detected in all developing lifestages indicating that TSWV is transstadially passed in vector thrips (Figure 5). Virus titers were high in larvae fed on TSWV infected plants and persisted through several molts and pupation. In 2 tests, our data suggest that the virus may replicate within F. occidentalis as the percentage of TSWV positive thrips decrease from larva 1 to larva 2, pupal stage, and show an increase at the late adult stage of development. We are continuing to pursue studies on virus multiplication comparing it with TEM and c-DNA probes studies.

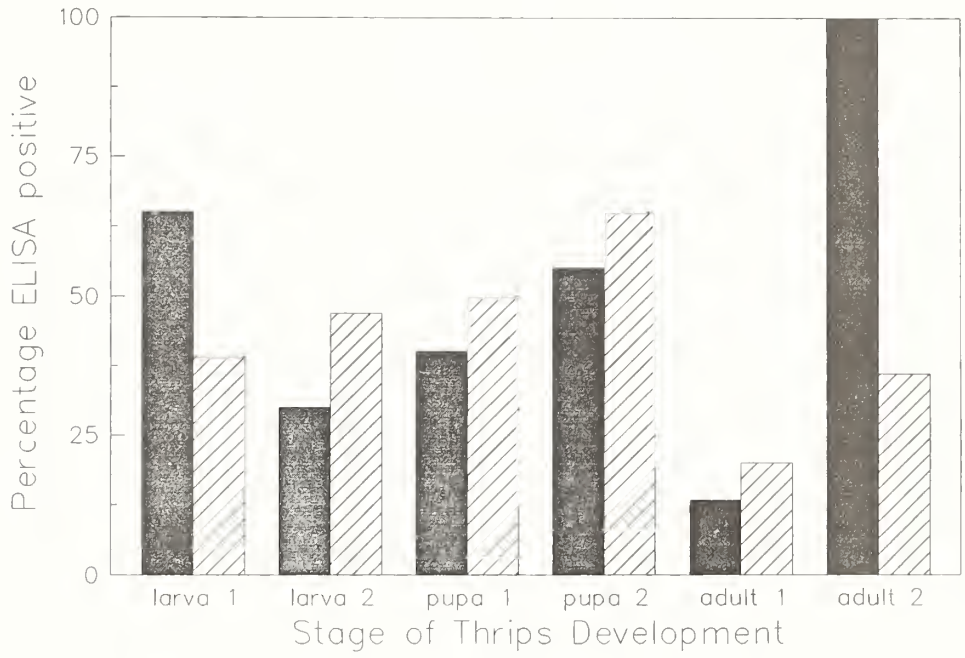

Figure 5. Persistence of TSWV in Frankliniella occidentalis in developing lifestages after acquisition access as larvae.

Comparisons between virus levels acquired by larval F. occidentalis and the percentage of ELISA positive adults in the developing population indicate a linear relationship (Figure 6). Larval thrips A405 nm values between 0 to 0.4 resulted in 20% of the adult 2 population being ELISA positive, 0.4 to 0.6 in 40%, 0.6 to 0.8 in 60%, and 0.8 to 1.1 in 80%. This correlation may explain Sakimura's (Sakimura, 1962) observation where an increase in the acquisition access period by larval thrips resulted in a concommitant increase in TSWV transmission. Further studies comparing the relationship of acquisition titers and transmission rates will determine the significance of this data.

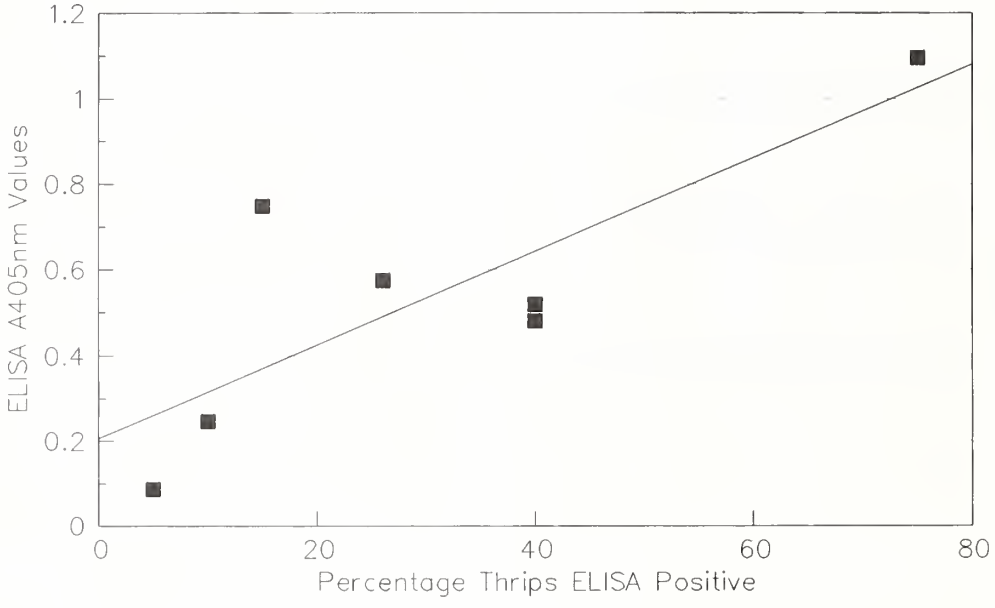

Figure 6. Relationship between virus levels detected in larval Frankliniella occidentalis and the percentage of ELISA positive developing adults.

TSWV was readily detected in 90% of the non-vector larval thrips, H. femoralis, when allowed access as larvae. However, the virus could not be detected in any of the developing adult 2 individuals tested.

Similar to the fate of TSWV in a non-vector thrips species, we observed a rapid decline in both virus titer and percentage of positive individuals after virus ingestion by adult F. occidentalis (Figure 7). TSWV was detected in 86% of the adults immediately after acquisition access feeding, declined to 18% one da after feeding, 5% after 2 da, 5% after 4 da, and 0% after 8 da. These data suggest that transmission may depend on retention and perhaps replication of the virus within thrips.

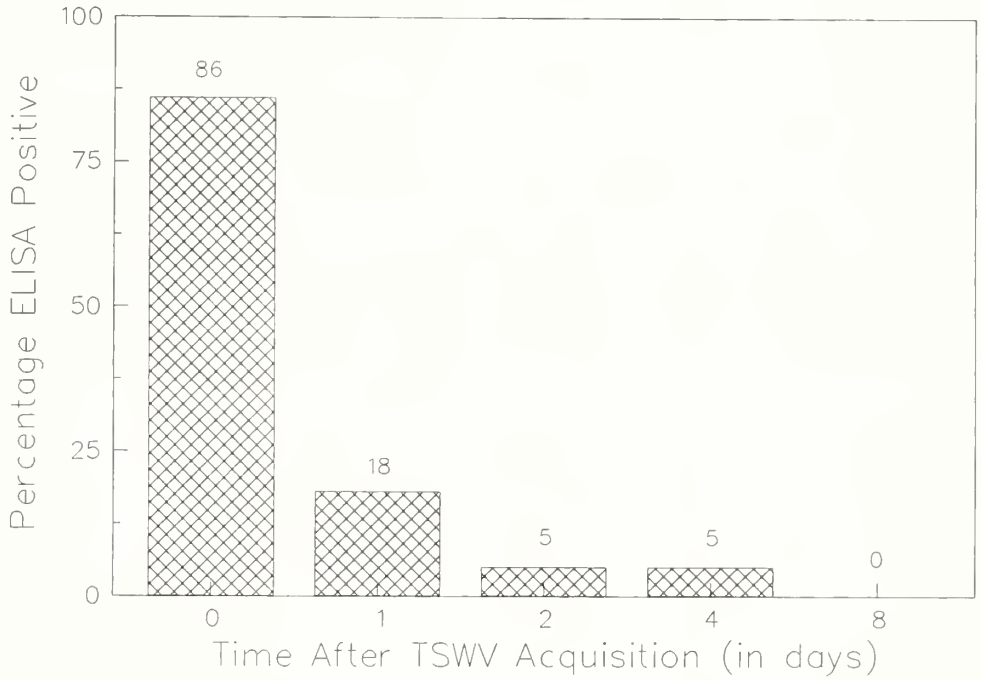

Figure 7. Persistence of TSWV in Frankliniella occidentalis after acquisition access as adults.

ELISA has been used effectively in addressing some of the fundamental questions relating to the virus-vector relationship. Our data show that thrips' ability to acquire virus from infected plants, a primary determinant of vector specificity for many plant viruses, clearly depends on a more complex mechanism than just thrips ability to ingest virus from TSWV infected plants. Although a high percentage of individuals, adult vector and non-vector thrips species, become ELISA positive when fed on TSWV-infected plants, the capability of vector species to pass TSWV transtadially, and retain the virus as adults are events necessary for effective transmission to occur. This work lays part of the foundation necessary for us to relate the ability of thrips to acquire and retain the virus with effective transmission to susceptible plant hosts.

REFERENCES

Amin, P. W., D. V. R. Reddy, and A. M. Ghanekar. 1981. Transmission of tomato spotted wilt virus, causal agent of bud necrosis of peanut, by Scirtothrips dorsalis and Frankiniella schultzei. Plant Dis. 65:663-665.

Bald, J. G., and G. Samuel. 1931. Investigations on "spotted wilt" of tomatoes. II. Australian Counc. Sci. Indus. Res. Bull. No. 54.

Best, R. J. 1968. Tomato spotted wilt virus. p. 65-145. In K. M. Smith and M. A. Lauffer (eds.), Advances in Virus Research. Vol. 13, Academic Press, New York.

Cho, J. J., R. F. L. Mau, R. T. Hamasaki, and D. Gonsalves. 1988. Detection of tomato spotted wilt virus in individual thrips by enzyme-linked immunosorbent assay. Phytopathology 78:1348-1352.

Cho, J. J., R. F. L. Mau, D. Gonsalves, and W. C. Mitchell. 1986. Reservoir weed hosts of tomato spotted wilt virus. Plant Dis. 70:1014-1017.

Gardner, M. W., C. M. Tompkins, and O. C. Whipple. 1935. Spotted wilt of truck crops and ornamental plants. Phytopathology. 25:17.

Gonsalves, D., and E. Trujillo. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.

Kobatake, H. 1984. Ecology and control of spotted wilt disease of tomato in Nara Prefecture. Proc. Kansai Plant Prot. Soc. 26:23-28.

Pittman, H. A. 1927. Spotted wilt of tomatoes. J. Aust. Counc. Sci. Ind. Res. 1:74-77.

Sakimura, K. 1963. Frankliniella fusca, an additional vector for the tomato spotted wilt virus, with notes on Thrips tabaci, another vector. Phytopathology 53:412-415.

Sakimura, K. 1962. The present status of thrips-borne viruses. p. 33-40. In K. Maramorosch (ed.) Biological Transmission of Disease Agents. Academic Press, New York.

Sakimura, K. 1946. Two species of thrips non-vectors of the spotted wilt virus. J. Econ. Entomol. 39:390-399.

Samuel, G., J. G. Bald, and H. A. Pittman. 1930. Investigations on "spotted wilt" of tomatoes. Aust. Counc. Sci. Ind. Res. Bull. 44. 64 pp.

Tanigoshi, L. K., and J. Y. Nishio-Wong. 1982. Citrus Thrips: Biology, Ecology and Control. USDA, ARS Tech. Bull. 1668. 17 pp.

Use of Strain-Specific Monoclonal and Polyclonal Antibodies to Detect Two Serotypes of Tomato Spotted Wilt Virus.

R. F. Davis, V. DeHerrera, L. Gonzales, and C. Sutula.

ABSTRACT

Distinct serotypes of tomato spotted wilt virus have been described and characterized from lettuce (TSWV-L) and Impatiens (TSWV-I). DAS-ELISA tests based on polyclonal antisera (PCA) produced against TSWV-L and TSWV-I are highly specific for homologous virus and show little cross reaction with the heterologous strain. Anti-TSWV-L monoclonal antibodies (MCA), produced in other laboratories, were evaluated and found to be highly specific for TSWV-L. These MCA's were effective in DAS-ELISA, for capture of TSWV-L, and for detection of TSWV-L captured by either anti-TSWV-L or TSWV-I PCA. TSWV-I was neither captured nor detected, by these MCA's. An improved TSWV-L test was developed, utilizing PCA for capture, and MCA for detection. This improvement resulted in lower background reactions to healthy tissue, and greater detection endpoints of sap from virus-infected plants.

Results of testing samples in our laboratory for both TSWV-L and TSWV-I over the past year, indicate that TSWV-L is more prevalent in field crops, and TSWV-I is more prevalent in greenhouse crops. However, both strain types have been identified in numerous vegetable and ornamental species from various locations throughout the United States.

INTRODUCTION

Tomato spotted wilt virus (TSWV) poses a significant threat to growers of ornamental, vegetable, and field crops (Lawson and Dienelt, 1990; Martins, 1989; Miller, 1989; Zitter et al., 1989). This virus has a very wide host range and is readily transmitted by certain thrip vectors (Ie, 1970). TSWV appears to have spread in epidemic proportions over the last several years, especially in North America.

Agdia, Inc., dedicated to quality testing products for the agricultural industry, has offered TSWV testing since 1986. The first TSWV test, introduced as TSWV but now known as TSWV-L, is based on an antiserum produced against a typical isolate of TSWV from lettuce (Gonsalves and Trujillo, 1986). This test is very important and effective in detection of TSWV-L and serologically related isolates. However, in 1987 we became aware that some isolates of TSWV, particularly those from impatiens, were not detected with this test.

Law and Moyer (1989, 1990) reported on the purification and characterization of an unusual TSWV variant from impatiens (TSWV-I). They reported that TSWV-I was serologically distinct from TSWV-L. Specifically, they found that the nucleoproteins of the two strains were not related by Western blot analysis with polyclonal antibodies. However, the G1 and G2 proteins were found to be related.

A new test for TSWV-I, based on a polyclonal antiserum developed against an impatiens isolate of TSWV (Law and Moyer, 1989) was introduced by Agdia in 1988.

Davis is a plant pathologist, DeHerrera and Gonzales are biologists, and Sutula is President, Agdia, Inc. 30380 County Rd. 6, Elkhart, Indiana 46530.

In a continuing effort to improve our tests for TSWV, we evaluated several monoclonal antibodies produced against TSWV-L (Hsu et al., 1990; Sherwood et al., 1989). The TSWV-L test has been modified through the use of a combination of polyclonal and monoclonal antibodies, resulting in increased sensitivity and specificity.

The purpose of this paper is to present the characteristics of our TSWV-L and TSWV-I tests, and to report the results of samples tested in our laboratory for TSWV over the past year.

MATERIALS AND METHODS

Virus isolates. TSWV isolates TOM, D, and GT ('L' serotype), and INC, GG, and SMITH ('I' serotype) were obtained from Jim Moyer (North Carolina State University). Other isolates were obtained from samples submitted to our laboratory for testing. Agdia isolate L062-1 came from tomato, and isolate K850-1 originated in impatiens.

Antibodies. Polyclonal antibodies produced against TSWV-L (Gonsalves and Trujillo, 1986) and TSWV-I (Law and Moyer, 1989) will hereafter be referred to as L-PCA and I-PCA, respectively. Monoclonal antibodies used in this evaluation were produced separately by Hsu et al. (1990) and Sherwood et al. (1989), and will be referred to generically as L-MCA.

ELISA methods. Procedures for the standard TSWV-L and TSWV-I tests were modified from Voller et al. (1976) and Clark and Adams (1977). Other methods will be described in Results section for individual tests. The standard buffer for sample extraction was PBST containing 2% polyvinylpyrrolidone-40, 0.02 M sodium azide, 0.01 M sodium sulfite, 4% fresh egg white, and 2% additional Tween-20, pH 7.4.

RESULTS

Specificity of TSWV-L and TSWV-I tests. The TSWV isolates we have tested are in two distinct serogroups. TSWV isolates TOM, D, GT, and L062-1 are detected by the TSWV-L test, but not by the TSWV-I test (Table 1). Similarly, TSWV isolates INC, GG, SMITH, and K850-1 are detected by the TSWV-I test, but not the TSWV-L test. In testing all combinations of antigens and polyclonal antibodies (L-PCA and I-PCA) for both capture and detection, TSWV-L can be captured only with the homologous sandwich of L-PCA and enzyme-conjugated L-PCA, and TSWV-I is detected only with the similar homologous sandwich of I-PCA antibodies (Table 2).

Characteristics of Improved TSWV-L test. The standard TSWV-L test was modified by the use of L-MCA to detect virus trapped by L-PCA. Following incubation of sample on the antibody coated plate, L-MCA was added for 1-3 hr. Alkaline phosphatase conjugated goat anti-mouse IgG+IgM was then added at a dilution of 1:2000 for 2-3 hr. Several monoclonal antibodies were evaluated in this format. All L-MCA's showed specificity for TSWV-L and many showed high sensitivity and low background as well. The results of two of these L-MCA's, designated A0796 and 208, are shown in Table 3.

These L-MCA were also capable of detecting TSWV-L trapped on I-PCA coated plates (Table 4). This indicates that antibodies present in I-PCA can capture TSWV-L. The reaction was not detected with enzyme conjugated L-PCA, but was detected with the more sensitive double-sandwich-indirect system using L-MCA and enzyme conjugated anti-mouse IgG+IgM.

These L-MCA were also capable of capturing TSWV-L but not TSWV-I, when used as the coating antibody in a double-sandwich assay.

TSWV-L trapped by L-MCA was detected by alkaline phosphatase conjugated L-PCA (Table 5). However, there was no reaction of the samples trapped on L-MCA coated plates, when the I-PCA conjugate was used (Table 6). This indicates that although I-PCA can trap TSWV-L, the same antibodies conjugated to alkaline phosphatase cannot bind to TSWV-L, at least in detectable quantity.

An experiment with buffers for extracting sap from infected plant tissues was conducted. Our standard extraction buffer was compared to a similar buffer containing 0.01 M sodium diethydithiocarbamate and 0.003 M ethylenediaminetetraacetate. The addition of these agents to this buffer resulted in a significant decrease in the sensitivity of the test (Fig. 1).

The test can be shortened by decreasing the incubation times for sample, MCA, and anti-mouse conjugate. Incubation times of 20 min were compared with 45 min, and the response was roughly double at 45 min incubation times (Fig 1). There was virtually no difference between incubation of reagents at 37 C and 4 C in this test (results not shown).

The dilution end point of samples in the improved TSWV-L test varied among samples, with a typical range between 1:4,000 and 1:400,000 (Fig 2) at 60 min substrate incubation.

Detection of TSWV. During the time period between March 1989 and April 1990, TSWV was detected in 284 samples from a variety of ornamental and vegetable crops (Table 7). These samples were submitted for TSWV analysis from 23 states in the United States and 1 province in Canada. Of the 258 samples from 24 ornamentals that tested positive for TSWV, 212 of these (82%) were positive for TSWV-I, 42 (16%) were positive for TSWV-L, and 4 (1.5%) were positive for both serotypes. Of the 26 samples from 5 vegetables that were positive for TSWV, 7 (27%) were positive for TSWV-I and 19 (73%) were positive for TSWV-L. Mixed infections were not detected in the vegetable samples.

DISCUSSION

Agdia's DAS-ELISA tests for TSWV-L and TSWV-I using polyclonal antibodies are very specific and show little cross reactivity with the heterologous serotype. However, results presented herein indicate that TSWV-L isolates can be trapped on plates coated with I-PCA. Although this trapped virus cannot be detected with enzyme-conjugated L-PCA, it can be detected with L-MCA, which are more sensitive. The I-PCA serum was produced mainly to nucleocapsid, but does show trace amounts of antibodies specific for the structural proteins G1 and G2 (Moyer, personal communication). The serological relationships of the structural proteins of TSWV-L and TSWV-I serotypes have been previously reported (Law and Moyer, 1989, 1990). We interpret our results to indicate that anti-TSWV-I G1 and/or G2 antibodies are binding TSWV-L isolates. That the nucleocapsids of TSWV-L and -I are not related (Law and Moyer, 1989, 1990) is supported by our findings that TSWV-I can only be detected in ELISA using a homologous system of anti-TSWV-I coating and conjugated antibodies. Monoclonal antibodies directed against the nucleocapsid of TSWV-L (Hsu et al., 1989; Sherwood et al., 1989) do not recognize TSWV-I.

Isolates testing positive for the TSWV-L serogroup were found in 73% of the vegetable samples and 16% of the ornamental samples, which tested positive for TSWV at Agdia. Isolates testing positive for the TSWV-I serogroup were found in 82% of the ornamental samples and 27% of the vegetable samples testing positive for TSWV. Mixed infections of both serotypes are very rare, and were detected in only 1.5% of the ornamental samples.

These samples were submitted by customers for analysis and do not represent an organized survey. However, the samples did come from a variety of plants and geographic locations and the results clearly indicate that both serotypes are found in ornamental and vegetable crops. Therefore, it is important to test for both serotypes of TSWV, in order to minimize the risk of false negative responses.

Our experience with extraction buffers indicates that ELISA detectability of the virus is inhibited when DIECA and EDTA are present. Optimum buffer components for extraction remains to be investigated, particularly to address the problems caused by the mucoid nature of impatiens sap.

We have found that ELISA detectability of the virus remains high for several days in sap extracts or unextracted tissue stored at 4 C, but decreases significantly in extracts or tissue which are stored at -20 C (unpublished data). We have also noted that sap extracts 'aged' at 4 C, produce higher reactions than those prepared fresh from the same sample.

References:

Clark, M. F., and A. N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay (ELISA) for the detection of plant viruses. J. Gen. Virol. 34:475-483.

Gonsalves, D., and E. E. Trujillo. 1986. Tomato spotted wilt virus in papaya and detection of the virus by ELISA. Plant Dis. 70:501-506.

Hsu, H. T., Y. C. Wang, R. H. Lawson, M. Wang, and D. Gonsalves. 1990. Splenocytes of mice with induced immunological tolerance to plant antigens for construction of hybridomas secreting tomato spotted wilt virus-specific antibodies. Phytopathology 80:158-162.

Law, M. P., and J. W. Moyer. 1990. A tomato spotted wilt-like virus with a serologically distinct N protein. J. Gen. Virol. 71:933-938.

Law, M. P., and J. W. Moyer. 1989. Physiochemical analysis of a serologically distinct tomato spotted wilt virus strain. (Abstr.) Phytopathology 79:1157.

Lawson, R. H., and M. M. Dienelt. 1990. TSWV: A diagnostic puzzle. Greenhouse Manager Jan:151-154.

Martins, J. A. 1989. They're invisible and incurable. Are viruses invincible, too? GrowerTalks March:40-42.

Miller, R. 1989. Identifying the most serious problem we face today - TSWV. GrowerTalks July:100-106.

Sherwood, J. L., M. R. Sanborn, G. C. Keyser, and L. D. Myers. 1989. Use of monoclonal antibodies in detection of tomato spotted wilt virus. Phytopathology 79:61-64.

Voller, A., A. Bartlett, D. E. Bidwell, M. F. Clark, and A. N. Adams. 1976. The detection of viruses by enzyme-linked immunosorbent assay (ELISA). J. Gen. Virol. 33:165-167.

Zitter, T., M. L. Daughtrey, and J. P. Sanderson. 1989. Tomato Spotted Wilt Virus. Fact Sheet Page 735.90 in: Vegetable/Horticultural Crops, Cornell Cooperative Extension, Cornell University, Ithaca, New York.

Table 1
Detectability of TSWV isolates in Agdia's tests for TSWV-L and TSWV-I, using polyclonal antibodies in DAS-ELISA.

Sample	Serotype	TSWV-L O.D.	TSWV-L Score	TSWV-I O.D.	TSWV-I Score
TSWV-TOM	L	0.989	+	0.025	-
TSWV-D	L	1.158	+	0.044	-
TSWV-GT	L	0.968	+	0.045	-
TSWV-L062-1	L	0.435	+	0.026	-
TSWV-INC	I	0.135	-	2.604	+
TSWV-GG	I	0.179	-	2.682	+
TSWV-SMITH	I	0.123	-	2.589	+
TSWV-K850-1	I	0.063	-	1.888	+
H-tobacco	-	0.103	-	0.022	-
H-tomato	-	0.037	-	0.018	-
H-gloxinia	-	0.074	-	0.025	-
Buffer	-	0.036	-	0.024	-

O.D. = optical density at 405 nm. Values are averages of 1:20, 1:80, and 1:320 (w:v) dilutions of sap.

Score: + = positive reaction, - = negative reaction.

Table 2
Detectability of TSWV isolates in Agdia's tests for TSWV-L and TSWV-I, using all combinations of polyclonal antibodies for coating and for enzyme-conjugated IgG in DAS-ELISA.

Sample	Serotype	anti-TSWV Coating/Conjugate Antibodies L/L O.D.	L/L Score	L/I O.D.	L/I Score	I/L O.D.	I/L Score	I/I O.D.	I/I Score
TSWV-D	L	1.385	+	0.061	-	0.177	-	0.046	-
TSWV-L062	L	0.347	+	0.018	-	0.045	-	0.020	-
TSWV-INC	I	0.099	-	0.068	-	0.086	-	3.000	+
TSWV-K850-1	I	0.038	-	0.027	-	0.024	-	1.789	+
H-tobacco	-	0.080	-	0.024	-	0.069	-	0.038	-
H-tomato	-	0.054	-	0.014	-	0.037	-	0.025	-
H-geranium	-	0.062	-	0.027	-	0.049	-	0.035	-
Buffer	-	0.059	-	0.032	-	0.051	-	0.036	-

Table 3
Results of DAS-Indirect ELISA using anti-TSWV-L polyclonal antibodies for capture and monoclonal antibodies for detection.

		Ascites					
		A0796		208		Preimmune	
Sample	Serotype	O.D.	Score	O.D.	Score	O.D.	Score
TSWV-TOM	L	3.000	+	3.000	+	0.112	-
TSWV-D	L	3.000	+	3.000	+	0.078	-
TSWV-GT	L	3.000	+	3.000	+	0.114	-
TSWV-L062-1	L	3.000	+	2.018	+	0.082	-
TSWV-INC	I	0.079	-	0.067	-	0.085	-
TSWV-GG	I	0.050	-	0.046	-	0.075	-
TSWV-SMITH	I	0.046	-	0.064	-	0.074	-
TSWV-K850-1	I	0.027	-	0.037	-	0.051	-
H-tobacco	-	0.035	-	0.047	-	0.066	-
H-tomato	-	0.038	-	0.045	-	0.099	-
H-gloxinia	-	0.075	-	0.052	-	0.142	-
Buffer	-	0.022	-	0.067	-	0.084	-

Table 4
Results of DAS-Indirect ELISA using anti-TSWV-I polyclonal antibodies for capture and monoclonal antibodies for detection.

		Ascites					
		A0796		208		Preimmune	
Sample	Serotype	O.D.	Score	O.D.	Score	O.D.	Score
TSWV-TOM	L	0.884	+	1.000	+	0.101	-
TSWV-D	L	1.210	+	1.071	+	0.079	-
TSWV-GT	L	0.640	+	0.687	+	0.072	-
TSWV-L062-1	L	0.200	-	0.096	-	0.096	-
TSWV-INC	I	0.055	-	0.047	-	0.064	-
TSWV-GG	I	0.069	-	0.020	-	0.063	-
TSWV-SMITH	I	0.030	-	0.056	-	0.079	-
TSWV-K850-1	I	0.034	-	0.014	-	0.066	-
H-tobacco	-	0.039	-	0.017	-	0.063	-
H-tomato	-	0.061	-	0.030	-	0.108	-
H-gloxinia	-	0.095	-	0.045	-	0.143	-
Buffer	-	0.041	-	0.038	-	0.079	-

Table 5
Results of DAS-ELISA using monoclonal antibodies for capture and alkaline phosphatase conjugated anti-TSWV-L polyclonal antibodies for detection.

		Ascites					
		A0796		208		Preimmune	
Sample	Reciprocal dilution	O.D.	Score	O.D.	Score	O.D.	Score
TSWV-D	200	1.049	+	1.465	+	0.037	-
TSWV-D	400	0.993	+	1.224	+	0.042	-
TSWV-D	800	0.947	+	1.272	+	0.024	-
TSWV-D	1600	0.788	+	1.058	+	0.023	-
TSWV-INC	200	0.033	-	0.032	-	0.022	-
H-tobacco	200	0.010	-	0.015	-	0.020	-
H-gloxinia	200	0.005	-	0.004	-	0.012	-
Buffer	-	0.010	-	0.010	-	0.007	-

Table 6
Results of DAS-ELISA using monoclonal antibodies for capture and alkaline phosphatase conjugated anti-TSWV-I polyclonal antibodies for detection.

		Ascites					
		A0796		208		Preimmune	
Sample	Reciprocal dilution	O.D.	Score	O.D.	Score	O.D.	Score
TSWV-D	200	0.048	-	0.023	-	0.000	-
TSWV-D	400	0.009	-	0.025	-	-0.001	-
TSWV-D	800	0.013	-	0.017	-	0.001	
TSWV-D	1600	0.006	-	0.014	-	0.001	-
TSWV-INC	200	0.035	-	0.043	-	0.027	-
H-tobacco	200	-0.002	-	0.005	-	0.005	-
H-gloxinia	200	0.010	-	0.007	-	0.005	-
Buffer	-	0.002	-	0.009	-	0.010	-

Table 7
Summary of samples testing ELISA-positive for TSWV at Agdia, Inc. from March 1989 through April 1990.

Sample	Type	Number of samples infected with: TSWV-I	TSWV-L	Both
ANEMONE	Ornamental	1	0	0
BABY'S BREATH	Ornamental	1	0	0
BALSAM	Ornamental	1	0	0
BEGONIA	Ornamental	35	0	0
CALADIUM	Ornamental	0	0	1
CHRYSANTHEMUM	Ornamental	0	11	0
CINERARIA	Ornamental	21	0	0
COLEUS	Ornamental	1	0	0
DAHLIA	Ornamental	1	3	0
EXACUM	Ornamental	1	0	0
FATSIA JAPONICA	Ornamental	0	1	0
GERANIUM	Ornamental	1	0	0
GESNARIAD	Ornamental	24	21	0
GLOXINIA	Ornamental	15	1	0
IMPATIENS	Ornamental	101	2	3
IVY GERANIUM	Ornamental	0	1	0
JASMINE	Ornamental	0	1	0
LOBELIA	Ornamental	1	0	0
PETUNIA	Ornamental	1	0	0
PORTULACCA	Ornamental	4	0	0
RANUNCULUS	Ornamental	1	0	0
STEPHANOTIS	Ornamental	0	1	0
SWEDISH IVY	Ornamental	1	0	0
VINCA	Ornamental	1	0	0
CELERY	Vegetable	1	0	0
EGGPLANT	Vegetable	1	0	0
PEPPER	Vegetable	1	7	0
TOMATO	Vegetable	3	12	0
WATERMELON	Vegetable	1	0	0
Number of isolates from ornamentals		212	42	4
Number of isolates from vegetables		7	19	0
Total isolates		219	61	4

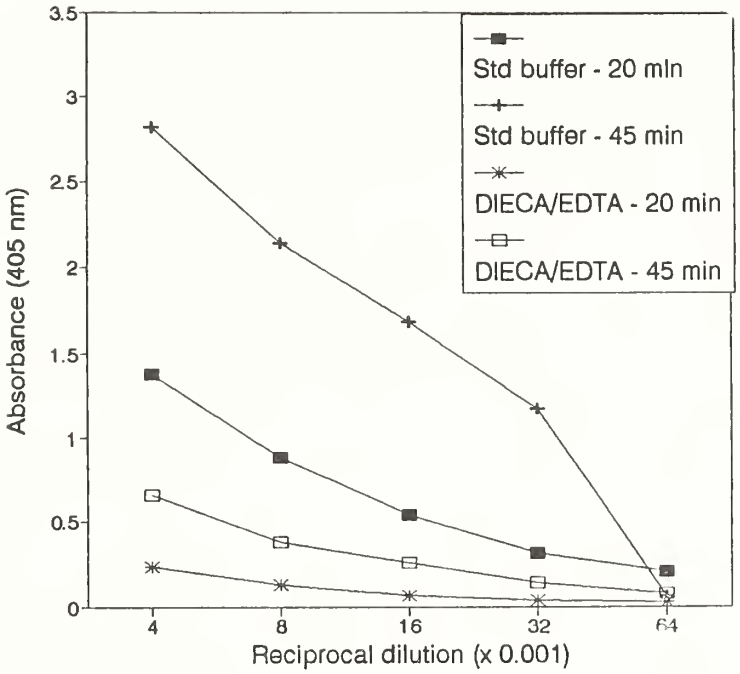

Figure 1
Effects of sample extraction buffer and reagent incubation time on Agdia's Improved TSWV-L Test. Double-antibody-sandwich indirect ELISA using L-PCA for trapping and L-MCA for detection. TSWV-L infected tissue extracted in standard buffer or one containing DIECA and EDTA, and diluted from 1:4,000 to 1:64,000. Incubation of sap, L-MCA, and alkaline phosphatase conjugated anti-mouse IgG+IgM, for 20 min or 45 min. Absorbance after 60 min substrate incubation.

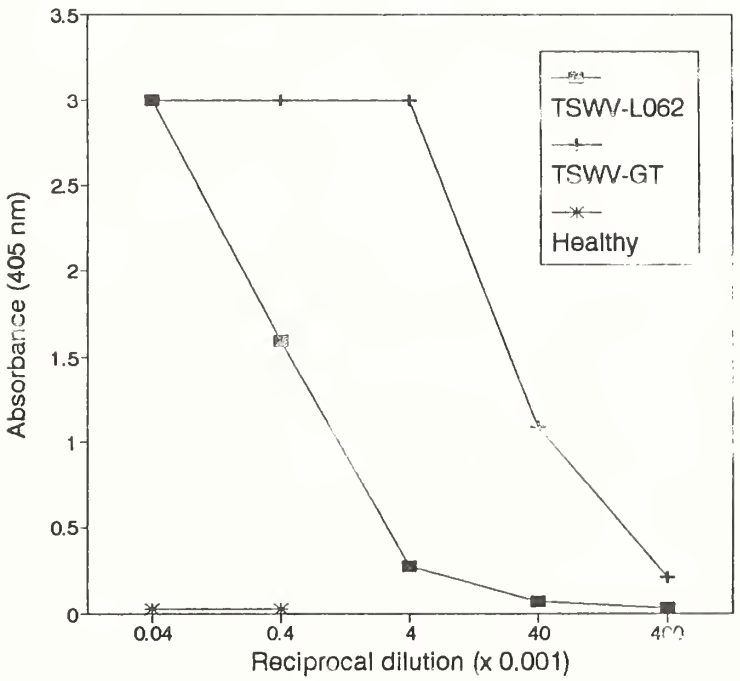

Figure 2
Effect of sample dilution on Agdia's Improved TSWV-L Test. Double-antibody-sandwich indirect ELISA using L-PCA for trapping and L-MCA for detection. TSWV-L062 infected tomato, TSWV-GT infected tobacco, and healthy tobacco diluted from 1:40 to 1:400,000. Absorbance measured after 60 min substrate incubation.

CONSEQUENCES OF AVOIDING TSWV IN A BREEDING STATION IN THE TEMPERATE ZONE

G. D. van Blokland

ABSTRACT

A balance of chemical and phytosanitary measures are required to minimize the occurrence of possible vectors of tomato spotted wilt virus in greenhouses. Sprays with insecticides were frequently made and after detection, sources of TSWV in and around the station were eradicted. A disease management program has been implemented to strictly segregate plant lots coming from different origins that may possibly be infected with TSWV.

INTRODUCTION

In the Netherlands the tomato spotted wilt virus (TSWV) was able to establish in the greenhouses of a few seed companies and government research stations. The virus was apparently introduced in material from various origins. Managing and controlling a virus which can easily be spread by thrips species in a greenhouse is difficult (Nameth et al., 1989). Since most growers are not familiar with the disease caused by TSWV and the symptoms sometimes develop slowly in certain hosts, the virus can spread rapidly in the greenhouse. Also the use of biological control programs in which thrips are not controlled, promoted the establishment of thrips populations. The western flower thrips, Frankiniella occidentalis, became epidemic after its introduction in many greenhouses into Europe. Moreover, this thrips is very hard to control with chemical treatments. Therefore, TSWV is becoming an important threat, especially in experiment stations with a continuous presence of different plant species. This problem is enhanced by the fact that the virus persists for life in the insect vector (Reddy and Wightman, 1988).

TSWV can be managed by adapting cultural practices that prevent or limit the activity of the vectors (Reddy and Wightman, 1988). According to Cho et al. (1989), it is important to understand the relationships between the crop, virus, insect vector, and resevoir hosts for the development of feasible management procedures. In Hawaii, disease management strategies are developed to minimize TSWV disease occurrence. Integrating these practices to reduce significantly diseases losses appeared possible (Cho et al., 1989). In this article, measures for controlling the TSWV problem are suggested and discussed.

TSWV identification and detection

TSWV is readily transmissible by sap inoculation from an infected plant, using abrasives. Therefore, initially a set of diagnostic species was used to identify TSWV (Ie, 1970). Testing suspicious plants Impatiens holstii give satisfactory results. In the case of Begonia tuberosa and Cyclamen persicum plants result in transfering TSWV to diagnostic species Tropaeolum majus, Petunia hybrida, Nicotiana rustica, Nicotiana glutinosa, and Vinca rosea were erratic.

A more rapid method and sensitive method to detect TSWV is a direct double antibody sandwich enzyme-linked immunosorbent assay (ELISA) (Gonsalves and Trujillo, 1986). The antiserum and the alkaline phosphatase-conjugated gammaglobulin were obtained from Dr. Peters (Dept. of Virology, LUW). The antiserum was prepared against the nucleocapsids of the virus. Testing of large numbers of plant samples has been scaled up and facilitated with a multigrinding mill by which 12 samples can be ground in the wells of a Costar microtiter plate 3596. The use of monoclonal antibodies (obtained from J. L. Sherwood, 1989) in the ELISA test gave some escapes. The ELISA technique was used to identify the TSWV sources both in plants and seeds in the research station. The use of diagnostic species and electron microscopy studies (by Dept. of Virology, LUW) in comparison with the ELISA test validate the reliability of the ELISA technique as a reliable, sensitive and rapid method for detecting TSWV. A direct relation between the type of symptoms and the presence of the virus by ELISA has been

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van Blokland is Project Leader, Phytopathology within Royal Sluis, R & D Department of Royal Sluis, P. O. Box 22, 1600 AA Enkhuizen, The Netherlands.

established for a number of our breeding crops. For example, small round necrotic lesions develop on young leaves and sepals of Impatiens infected with TSWV.

We do not have a technique at our station for detection of the virus in symptomless carriers. However, we used several methods for raising the virus level in plant parts by inducing a wound tissue reaction by cutting leaves or stems (Table 1) and by sampling the tissue after fruitset (Tomato). For symptom expression in Cyclamen we lowered the temperature to 12°C (Allen and Matteoni, 1988). Tubers of Dahlia and Cyclamen were sampled for detection of TWSV.

The methods, mentioned in Table 1, were only applied for a limited number of species. Research with more species has to be continued. For Cyclamen and Lycopersicon no symptoms were observed in young plants. Symptoms in Lycopersicon developed at about two months for Cyclamen with a temperature regime of 12°C. In Cyclamen the virus has a long latent period (Allen and Matteoni, 1988). During this latent period ELISA testing gave erratic results and the undetected spread of the disease continued. Sampling of wound tissue in Lycopersicon and Osteospermum, which tissue contained a higher virus content (pers. comm. Peters), did not result in earlier detection of infected material. The screening of tubers of Cyclamen and Dahlia offers the possibility to detect the presence of TSWV during the storage period, before virus spreading occurs.

TABLE 1. Methods for raising the virus concentration and/or stimulating symptom development in plants or certain crops suspected of TSWV infection.

	Aging of plant	Woundtissue	Temperature	Tuber
CROP				
Lycopersicon	X	X		
Osteospermum		X		
Cyclamen	X		X	X
Dahlia				X

Chemical control of the vector

The most prevalent thrips species in our glasshouses in the Netherlands is F. occidentalis. The thrips populations were effectively controlled with biweekly treatment with Dichlorvos. The application of this insecticide appeared to have toxic effects on the plants of some species. Also the production of seed as well as their quality was affected. To prevent resistance to insecticides a new control scheme was introduced. Four different chemicals including Methomyl, Propoxur, Oxamyl and the synthetic pyrethroid, Bifentrim, are sprayed alternatly. One type of chemical is applied one time a month. It is also evident that these sprays cause less crop damage. Thrips population have been reduced to less than one thrips per square meter. A low volume mist blower is used for the application of chemicals. Drop sizes of 1-20 micron are obtained.

A complete eradication could only be obtained by removing the whole crop from the greenhouse, raising the temperature to 40°C, and applying Dichlorvos sprays three times within a week.

Controlled introduction of new seed and plant material

Quarantine measures have been implemented to prevent the introduction of infected material in the research station.

Transmission of TSWV through seeds

The question whether TSWV can be transmitted through seeds has often been raised. Jones (1944) reported results showing that TSWV could be transmitted through Cineraria and tomato seeds. Crowley (1957) reports 1% infection via

tomato seeds. He states that the virus particles could be detected in the testa but not in the embryo. Most viruses that can be detected in the seed coat are not transmitted to the next generation.

Seed transmission of TSWV was tested in two experiments. The seed lots for this transmission study were harvested from different TSWV infected tomato plants and the seeds were rated positive for virus presence by ELISA. Two seed lots from infected plants and one seed lot from a healthy plant were germinated in a quarantine compartment. No other source of infection was present in this compartment. Half of the healthy seedings were placed in another TSWV-free quarantine cell.

TABLE 2. Seed transmission of TSWV in tomato seed

Seedlot	Infected	No. of seeds	TSWV infected plants
EXP. 1			
89-Ly-10	Yes	30	1
89-Ly-12	Yes	30	0
89-Ly-15	No	30	0
EXP. 2			
89-Ly-12	Yes	50	0
89-Ly-13	Yes	50	7
89-Ly-15	No	50	0

Screening for TSWV infected plants in the different seed lots started in the third leaf stage in wound tissue. All plants were checked weekly for TSWV infections. The final results of the experiment are shown in Table 2.

In the first experiment with infected tomato seeds it took two and a half months before the first symptoms appeared. The tomato plant was in the third cluster stage. ELISA reactions, which were rated visually, were strongly positive. In the second trial TWSV plants were detected after two months, although clusters were not yet developed. The typical tomato bronzing spots became visible. Bright, yellow sticky strips were placed just above the crop canopy to monitor the thrips. During the experiment no thrips was observed in the quarantine compartments.

To locate the virus in the seed the tomato seeds were dissected and the embryo/endosperm part separated from the seed coat. For detection of the virus in the seed, the whole seed was used in the ELISA test. After partition of the seed in testa and embryo, both parts were separately tested. From the infected seeds in all testa, TSWV was present. However, TSWV was detected with ELISA in only 10% of the sectioned embryo/endosperm. The seed transmission studies are only based on only two experiments. More experiments are necessary to obtain reliable statistical results.

Plants

Plant material introduce in a breeding or plant research station has to be placed in quarantine and thoroughly treated against thrips. After an observation period and testing every suspected plant with ELISA, the non-infected plants can be placed near the other crops. During their cultivation period the introduced crops should be followed more closely.

Management strategies

A number of phytosanitary measures and control procedures are required to reach a significant reduction of disease losses. Integration of the knowledge about virus, virus source plants, insect vectors and the normal hygenic and chemical control measures will reduce the damage by the virus to a manageable level. The first step is to get rid of all infected plants. However, plants may harbor virus that do not show any symptoms. The techniques mentioned in this article (aging, wound tissue, temperature) will be applied. In case of

detection of one infected plant all surrounding plants should be followed closely. In addition, weeds must be eliminated in and around the greenhouse. The second step is the control of thrips in the crops, but also outside the greenhouse.

The main guidelines for managing a TSWV free crop program are:

- Every plant lot introduced in the research station has to pass a quarantine procedure and has to be made free of all thrips.

- Every seed lot from crops possibly infected with TSWV of which seed transmission has been proven, will be treated prior to the introduction in the station. Research on temperature treatment of seeds is initiated.

- Separation of plant material possibly infected with TSWV must be separated from healthy plants within a crop.

- Crops should be segregated on the basis of cultivation and chemical control methods.

An overview to survive the threat

The last four years TSWV has become an important threat to breeding and research stations in Northwest Europe. The main reason is the increase in populations of the western flower thrips, which are difficult to control. Another reason is the introduction of seed and plant material from areas with TSWV epidemics. Besides, we have to deal with a vector and a virus which infect a broad range of species. An advantage in the temperate zone to the sub-tropical and tropical research stations is the break in the cycle of the vector and the TSWV reservoir host outside the greenhouse. This offers an opportunity to make the station free of the virus vector in combination with a number of hygenic measures in the greenhouse. The approach of strict segregation between different plant lots reqires an expensive construction program for new glasshouse compartments. Both for the breeding work and for the delivery of absolute TSWV free material, the company Royal Sluis feels obliged to prevent spread of the tomato spotted wilt virus by all possible methods.

LITERATURE CITED

Allen, W. R., and J. A. Matteoni. 1988. Cyclamen ringspot: Epidemics in Ontario greenhouses caused by the tomato spotted wilt virus. Can. Jour. of Botany 10:41-46.

Cho, J. J., Mau, R. F. L., German, T. L., Hartmann, R. W., Yudin, L. S., Gonsalves, D., and R. Providenti. 1989. A multidisciplinary approach to management of tomato spotted wilt virus in Hawaii. Plant Disease 73(5).

Crowley, N. C. 1957. Studies on the seed transmission of plant virus diseases. Austr. J. Biol. Sci. 10:449-464.

Gonsalves, D. 1986. Tomato spotted wilt virus in Papaya and detection of the virus by Elisa. Plant Disease 70:501-506.

Ie, T. S. 1970. Tomato spotted wilt virus. CMI/AAB Descriptions of plant Viruses n.39

Jones, L. K. 1944. Streak and mosaic of Cineraria. Phytopathology 34:941-953.

Nameth, S. T., Lindquist, R. K., and C. C. Powell. 1989. Tomato spotted wilt virus: a serious threat to greenhouse crops. Extension Plant Pathology; The Ohio State University, 2021 Coffey Road, Columbus, Ohio 43210.

Reddy, D. V. R., and J. A. Wightman. 1988. Tomato spotted wilt virus: thrips transmission and control. Adv. Disease Vector Res. Vol. 5. K. F. Harris, ed.

BEHAVIOR OF TOMATO SPOTTED WILT VIRUS FOLLOWING INTRATHORACIC INOCULATION INTO THE MOSQUITO TOXORHYNCHITES AMBOINENSIS

Min Wang, Carl J. Mitchell, Charles H. Calisher, and Dennis Gonsalves

ABSTRACT

Tomato spotted wilt virus (TSWV) is morphologically, molecularly and structurally similar to viruses of the family Bunyaviridae. Six-day-old adult Toxorhynchites amboinensis mosquitoes were inoculated with purified TSWV or with cucumber mosaic virus (CMV), a member of the cucumovirus group which served as a negative control. At intervals after injection, individual mosquitoes were ground in buffer and aliquots were inoculated onto a TSWV local lesion host or were kept at -20 C for serological and nucleic acid hybridization tests. Infectious virus was not detected in any of the injected insects during the five week test period. However, high levels of TSWV antigen were detected in these mosquitoes by ELISA for at least a week after injection. TSWV antigen concentration began to decrease thereafter, but remained at detectable levels for as long as five weeks after injection. In contrast, CMV coat protein concentrations decreased greatly within 15 min after injection as determined hy ELISA, and this protein could not be detected 12 hr after injection. Hybridization experiments with cDNA to detect TSWV RNA in injected mosquitoes gave inconsistent results.

INTRODUCTION

Tomato spotted wilt virus (TSWV), the sole member of the TSWV group, and viruses of the family Bunyaviridae have similar particle size, morphology and number of nucleic acid and protein components (Milne and Francki, 1984). Viruses in the family Bunyaviridae have membrane bound isometric particles 90-100 nm diameter. The genomic RNA of bunyaviruses is negative sense, and has three ssRNAs of 2.2-4.9 $X10^6$ (L), 1.0-2.3 $X10^6$ (M) and 0.3-0.8 $X10^6$ (S) MW (Bishop, 1985). TSW virions are membrane bound spherical particles 70-90 nm in diameter. TSWV has a divided genome of three ssRNAs with molecular weights of $2.7X10^6$ (L), $1.7X10^6$ (M) and $1.1X10^6$ (S) (Verkleij et al., 1982). Recent evidence suggests that TSWV M-RNA is negative sense (de Haan et al., 1989a).

Viruses in the Phlebovirus genus (family Bunyaviridae) have a nucleocapsid protein (20-30$X10^3$ daltons), two glycoproteins (G1, 60-70$X10^3$ daltons and G2, 50-60$X10^3$ daltons) and a large protein (150-200$X10^3$ daltons), believed to be a transcriptase (Bishop, 1985). On the other hand, the structural proteins of TSWV include a nucleocapsid protein ($27X10^3$ daltons) and three glycoproteins (52, 56 and $78X10^3$ daltons) (Mohamed, 1981, Tas et al. 1977). Both groups of viruses are transmitted by arthropods. Bunyaviruses replicate in and are transmitted by mosquitoes and other arthropods, TSWV is transmitted by thrips and can also be transmitted mechanically.

Milne and Francki (1984) have suggested that TSWV be considered a possible member of the Bunyaviridae based on published data about these viruses. The N-terminal 80 amino acids of the predicted gene product of TSWV M-RNA show 25% similarity with the NSm protein encoded by the M-RNA of Rift Valley fever virus in the Phlebovirus genus. In addition, there are similarities in the genome terminal structure of TSWV and hunyaviruses (de Haan et al. 1989 a,b). To determine whether TSWV replicates in mosquitoes, we injected TSWV into T. amboinensis.

Wang and Gonsalves, Plant Pathology Department, Cornell University, NYSAES, Geneva, NY 14456; Mitchell and Calisher, Division of Vector-Borne Infectious Diseases, Center for Infectious Diseases, Centers for Disease Control, Public Health Service, US Department of Health and Human Services, Fort Collins, CO 80522

MATERIALS AND METHODS

Virus preparations

Tomato spotted wilt virus (BL isolate) (Wang and Gonsalves, 1990) was purified from Datura stramonium leaves 12 days after inoculation. Virus was separated in a sucrose gradient and high speed centrifuged (65,000 X g) to pellet the virus; this preparation was called TSWV-final (TSWV-F). According to a virus absorption profile at A_{254} nm, the final concentration of TSWV-F was 20 O.D./ml. The original virus preparation from the sucrose gradient (TSWV-S) was used as another inoculum. The calculated concentration was about 1.7 O.D./ml. TSWV-S was suspended in 0.01 M Na_2SO_3 and approximately 20% sucrose. Healthy D. stramonium leaf tissue was treated in the same way and the final product was diluted in a similar amount of buffer as was done with infected tissue. Both virus and healthy preparations were resuspended in 0.01 M Na_2SO_3, pH 7.0. Cucumber mosaic virus (CMV) China strain (4 mg/ml) (Kearney, 1989) was used in the experiment as a negative control.

Injection of mosquitoes

Toxorhynchites amboinensis mosquitoes were selected for inoculation of TSWV because many animal viruses have been shown to replicate in them after intrathoracic inoculation (Rosen, 1981, Zeller and Mitchell, 1989). Six-day-old adult T. amboinensis were injected and subsequently reared at 25-27 C with 10% sucrose as the food source. Injections of TSWV-S and TSWV-F were initiated one hour and four hours after virus purification, respectively. Infectivity of the virus preparations were tested at the beginning and at the end of the injection period by inoculating susceptible Chenopodium quinoa plants. Preparations were injected (about 0.5 ul/insect) into mosquitoes in the membranous area of the thorax using glass needles (Rosen and Gubler, 1974). Three mosquitoes were injected at each time point for virus infectivity and concentration tests and 12 points were chosen altogether for each virus preparation.

Assays of injected mosquitoes for virus infectivity and concentrations

At various times after injection, individual mosquitoes were ground in solvent 4 buffer (0.03 M KH_2PO_4, 0.06 M K_2HPO_4, and 0.01 M Na_2SO_3, pH 7.2), 0.5 ml buffer/insect. Part of the extract was saved at -20 C for virus titrations at the end of the experiment. The remainder was inoculated onto C. quinoa at 1:5 and 1:100 dilutions to determine virus infectivity. Five weeks after injection, all of the frozen mosquito extracts were thawed and tested by double sandwich direct ELISA (Clark and Adams, 1977) with anti-TSWV rabbit antibody and by dot blot hybridization (Palukaitis, 1984). The probe used in the hybridization test was either complementary DNA made from TSWV RNA by random priming, according to the procedure of Sambrook et al. (1989), or radiolabeled mixtures of inserts of cloned TSWV S-RNA and M-RNA (Wang and Gonsalves, unpublished). The procedure for the latter was described by Sambrook et al. (1989).

RESULTS

Bioassays

Both TSWV-S and -F and CMV preparations were highly infectious at the end of the injection period when inoculated in undiluted form (about 200 local lesions formed in 25 cm^2 area) onto plants. Extracts of inoculated mosquitoes were not infectious on C. quinoa. The calculated dilutions of the virus in these extracts were 1:5,000 and 1:100,000.

Serological assays

Results of ELISA studies of the persistence of virus coat proteins after inoculation of virus into mosquitoes are summarized in Fig. 1. TSWV protein was detected at a high level for up to one week in mosquitoes injected with either TSWV-S or -F. Thereafter, the concentration of the coat protein decreased. In contrast, CMV coat protein concentration

decreased greatly within 15 minutes after injection, and was below the level of detection 12 hrs after injection. ELISA reactivities of TSWV injected mosquitoes decreased 15 to 30 min after injection and then increased, and in the case of the cohort inoculated with a 1:25 dilution of TSWV-F, appeared to stabilize for about one week.

Dot blot hybridization

In one experiment using cDNA synthesized from TSWV-RNA, the concentration of TSWV RNA increased between 30 min and 12 hrs after inoculation, as estimated by the intensity of reactions in dot blot hybridization. Positive reactions were not observed in extracts of mosquitoes incubated for 48 hours to three weeks. TSWV RNA could be detected again in the mosquitoes after four (three of four mosquitoes tested) and five (three of four mosquitoes tested) weeks incubation. However, we were not able to obtain any positive reactions in two subsequent experiments.

DISCUSSION

We could not detect infectious TSW virus, as determined by bioassay, after virus was inoculated into the hemocoel of the mosquito T. amboinensis. However, because of the extremely high dilutions of the virus in the mosquito, combined with our relatively insensitive bioassay technique, low levels of virus replication would probably not have been detected. Mosquitoes are more susceptible to infection by arboviruses after parenteral injection than they are by feeding (Chamberlain and Sudia, 1961). Perhaps inoculating cultures of mosquitoes cells would give a more definitive answer to the question of TSWV replication in mosquitoes.

It appears that TSWV coat protein concentration remained at a relatively high level inside the mosquitoes for about a week. Calisher (unpublished data) has observed that TSW virus protein persists in C6/36 cells (a mosquito cell line) for about five days. In contrast, CMV was not detected after inoculation into mosquitoes. The mechanism by which TSWV coat protein persists inside T. amboinensis is unknown. It is likely that the coat protein detected by ELISA was not from intact virus particles. It is known that the antibodies we used in these ELISAs can react with denatured TSWV coat proteins (Wang and Gonsalves, 1990). We have no explanation for the "dip" of ELISA reactions of TSWV injected mosquitoes around 15 min after injection.

Although the dynamics of TSWV RNA persistence in mosquitoes in one experiment was very interesting, the phenomenon was not observed in subsequent experiments. The result from that experiment may simply be the exception. In summary, TSWV coat proteins persists for about a week inside injected T. amboinensis mosquitoes, but infectious TSWV apparently does not.

REFERENCES

Bishop, D.H.L. 1985. Replication of Arenaviruses and Bunyaviruses. In: Virology (Fields, B.N., D.M. Knipe, R.M. Chanock, J.L. Melnick, B. Roizman, and R.E. Shope, ed.), pp. 1083-1110. Raven Press, New York.

Chamberlain, R.W., and W.D. Sudia. 1961. Mechanism of transmission of viruses by mosquitoes. Ann. Rev. Entomol. 6:371-390.

Clark, M.F., and A.N. Adams. 1977. Characteristics of the microplate method of enzyme-linked immunosorbent assay for the detection of plant viruses. J. Gen. Virology 34:475-483.

de Haan, P.T., Wagemakers, L., Goldbach, R., and Peters, D. 1989a. Tomato-spotted wilt virus, a new member of the Bunyaviridae? In: Genetics and pathogenicity of negative strand viruses. (D. Kolakofsky and B.W.J. Mahy, ed.), pp. 287-291. Elsevier, Amsterdam.

de Haan, P., Wagemakers, L., Peters, D., and R. Goldbach. 1989b. Molecular cloning and terminal sequence determination of the S and M RNAs of tomato spotted wilt virus. J. Gen. Virology 70:3469-3473.

Kearney, C.M. 1989. Molecular, ecological, and transgenic studies of cucumber mosaic virus and its satellite RNA. Ph.D. dissertation. Cornell University, 123 pp.

Milne, R.G., and R.I.B. Francki. 1984. Should tomato spotted wilt virus be considered as a possible member of the family Bunyaviridae? Intervirology 22:72-76.

Mohamed, N.A. 1981. Isolation and characterization of subviral structures from tomato spotted wilt virus. J. Gen. Virology 53:197-206.

Palukaitis, P. 1984. Detection and characterization of subgenomic RNA in plant viruses. In: Methods in Virology (K. Maramorosch and H. Koprowski, ed.), Vol. VII, pp. 259-317. Academic Press, Orlando, Florida.

Rosen, L. 1981. The use of Toxorhynchites mosquitoes to detect and propagate dengue and other arboviruses. Amer. J. Trop. Med. Hyg. 30:177-183.

Rosen, L., and D. Gubler. 1974. The use of mosquitoes to detect and propagate dengue viruses. Amer. J. Trop. Med. Hyg. 23:1153-1160.

Sambrook, J., E.F. Fritsch, and T. Maniatis. 1989. Molecular cloning, a laboratory manual. Second edition. Cold Spring Harbor Laboratory Press.

Tas, P.W.L., Boerjan, M.L., and D. Peters. 1977. The structural proteins of tomato spotted wilt virus. J. Gen. Virology 36:267-279.

Verkleij, F.N., P. de Vries, and D. Peters. 1982. Evidence that tomato spotted wilt virus is a positive strand. J. Gen. Virology 58:329-338.

Wang, M., and D. Gonsalves. 1990. ELISA detection of various tomato spotted wilt virus isolates using specific antisera to structural proteins of the virus. Plant Disease 74:154-158.

Zeller, H.G., and C.J. Mitchell. 1989. Replication of certain recently classified viruses in Toxorhynchites amboinensis mosquitoes and in mosquito and mammalian cell lines, with implications for their arthropod-borne status. Res. Virol. 140:563-570.

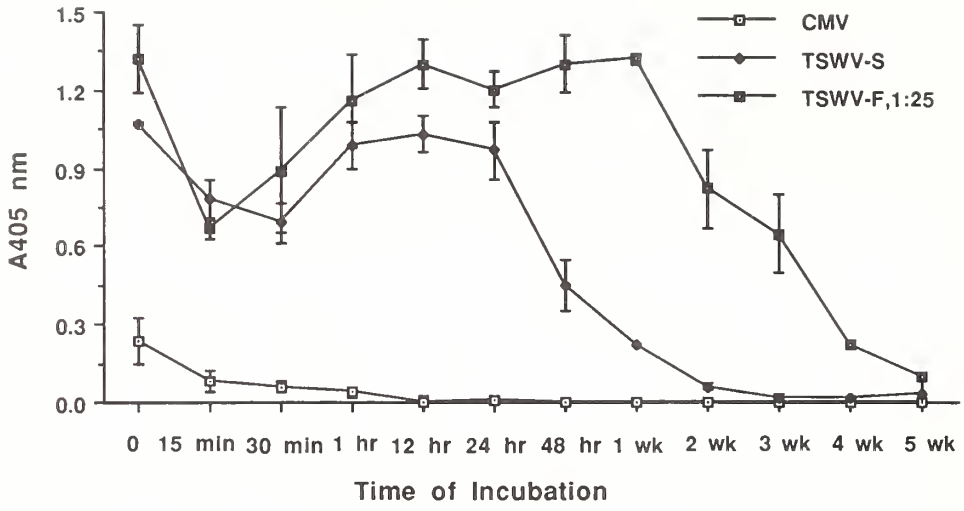

Figure 1

Double sandwich direct ELISA reactions of injected T. amboinensis mosquito extracts with TSWV sucrose gradient preparation (TSWV- S), final concentrated TSWV preparation (TSWV-F) (at 1:25 dilution), and with cucumber mosaic viruses (CMV) during various times of incubation. Each data point represents the average of ELISA readings of three or five mosquitoes. The hash marks represent the range of standard error.